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A Comparative Study of Behavior in Neonate Gartersnakes, Thamnophis butleri and T. radix (Colubridae), in an Area of Potential Hybridization

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To the Graduate Council:

I am submitting herewith a thesis written by Lauren Elizabeth Kirby entitled "A Comparative Study of Behavior in Neonate Gartersnakes, *Thamnophis butleri* and *T. radix* (Colubridae), in an Area of Potential Hybridization." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Arts, with a major in Psychology.

Gordon M. Burghardt, Major Professor

We have read this thesis and recommend its acceptance:

Arthur C. Echternacht, Todd M. Freeberg

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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We have accepted this thesis
and recommend its acceptance:

Arthur C. Echternacht

Todd M. Freeberg

Accepted for the Council:

Anne Mayhew
Vice Chancellor and
Dean of Graduate Studies

(Original signatures are on file with official student records.)

A Comparative Study of Behavior in Neonate Gartersnakes, *Thamnophis butleri* and
T. radix (Colubridae), in an Area of Potential Hybridization

A Thesis Presented for the
Master of Arts Degree
The University of Tennessee, Knoxville

Lauren Elizabeth Kirby
August 2005

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Abstract

An isolated population of Butler's gartersnake, *Thamnophis butleri* (Colubridae), in southeastern Wisconsin has recently been listed as Threatened by the Wisconsin Department of Natural Resources. One of the possible reasons for the decline of *T. butleri* in Wisconsin is pressure from a closely related species, the plains gartersnake, *T. radix*. The possibility of hybridization between *T. butleri* and *T. radix* has received recent attention because *T. butleri* is threatened in the area where the hybridization may be occurring. This study addresses the issue of hybridization by studying the behavior of neonatal *T. butleri* born to females originating from areas where the ranges of the species overlap (southeastern Wisconsin), as well as from areas outside the range of *T. radix* (Michigan and northern Ohio). Neonatal *T. radix* from northern Illinois were used as a *T. radix* "control". Pregnant females were collected from 4 counties in southeastern Wisconsin, with the southernmost county closest to the range of *T. radix*. I examined antipredator behavior, prey chemosensory responses, prey preference, and morphological size traits.

Several hypotheses have been put forth as to the interactions that occur when two species are sympatric, including character displacement, the importance of local ecology, and gene flow. Morphological and genetic evidence support the hypothesis of gene flow between *T. butleri* and *T. radix*, but this hypothesis has yet to be tested with only behavioral data. I had several predictions concerning the hypothesis of hybridization in these species: (1) the behaviors of *T. butleri* in Wisconsin would differ from the behaviors of Michigan and Ohio *T. butleri* in the direction of being more like *T. radix*; (2) the behaviors of *T. butleri* from the different counties and populations in Wisconsin

would differ from one another; (3) *T. butleri* from the northern part of the Wisconsin range, further removed from the range of *T. radix*, would show behaviors more like Michigan *T. butleri* and less like *T. radix* than would *T. butleri* from the southern part of their range in Wisconsin.

Michigan and Ohio *T. butleri* were heavier and longer than Wisconsin *T. butleri*, thus making the Wisconsin snakes less like *T. radix*. Populations within southeastern Wisconsin differed, and snakes from the population closest to *T. radix* were greater in length and mass than snakes from the population furthest from the range of *T. radix*. Snakes from Wisconsin were found to be more similar in body condition to *T. radix* than to Michigan and Ohio *T. butleri*.

Differences were found in antipredator behaviors across the populations of *T. butleri* from southeastern Wisconsin, with snakes from the population closest to the range of *T. radix* striking more frequently and therefore more similar to the *T. radix* studied than to Michigan and Ohio *T. butleri*. *Thamnophis butleri* from Michigan and Ohio were more likely to flee than *T. butleri* from southeastern Wisconsin. Within Wisconsin populations, snakes further removed from *T. radix* were more likely to flee, and were therefore more similar to Michigan and Ohio *T. butleri*.

Chemosensory preferences of the snakes also differed among the populations of *T. butleri* from southeastern Wisconsin. Snakes from the southernmost population showed a chemosensory preference for fish over worms, whereas snakes from the northern part of the range showed a chemosensory preference for worms over fish. Hence, the behaviors of *T. butleri* from the southern part of their range in Wisconsin, closer to the range of *T. radix*, are more similar to *T. radix* than are the behaviors of *T.*

butleri elsewhere in their range. The implications of these findings for the conservation and genetic study of Wisconsin *T. butleri* are discussed.

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1. INTRODUCTION

General Introduction

Research in animal behavior has led to important discoveries pertaining to evolution and the diversity of life. Behavior research has been conducted with a wide variety of animals and encompasses a diverse number of questions that can be organized around several major themes, including causation, function, ontogeny, evolution, and experience (Tinbergen 1963; Burghardt 1997).

Snakes are an informative group with which to study behavior due to their atypical feeding habits, mode of locomotion, and general lack of parental care. They also inhabit many regions of the world, and exhibit extreme differences in body size and shape, habitat preference, feeding behavior, and other evolutionary adaptations.

The genus *Thamnophis* (Colubridae), encompassing 30 known species, has been described as representative of terrestrial snakes in general because most members of the genus display typical size, shape, habits, and behavior (Rossman et al. 1996). Due to the availability of many species in the wild and easy maintenance in the laboratory, snakes of the genus *Thamnophis* have been studied more than any other group of snakes (Rossman et al. 1996). *Thamnophis* species consume a variety of prey types in the wild, and the genus is comprised of both prey generalists and prey specialists.

Butler's gartersnake, *Thamnophis butleri*, is found from central Ohio and central Indiana northward through eastern Michigan and the extreme southern tip of Ontario, Canada, with a geographically isolated population occurring in extreme southeastern Wisconsin (Rossman et al. 1996, see Figure 1). The population in southeastern

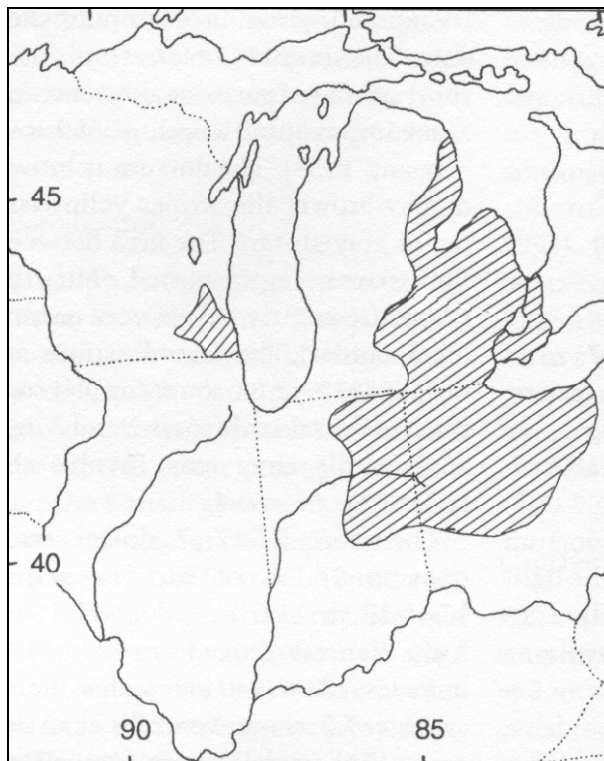


Figure 1. Accepted range of *T. butleri* in the midwest of the USA and Canada (from Rossman et al. 1996).

Wisconsin was listed as Threatened by the Wisconsin Department of Natural Resources in 1997 and is the focus of this study.

Although habitat destruction and fragmentation are involved, an additional possible reason for the decline of *T. butleri* in this area is pressure from a closely related species, *T. radix*, the plains gartersnake. This species is found southeastward from southern Alberta, Canada to northeastern New Mexico and the Oklahoma panhandle, then eastward through the Great Plains to southern Wisconsin, northern Illinois, and northwestern Indiana, with a remnant population in Ohio (Rossman et al. 1996). Of interest here is the part of the range of *T. radix* that abuts with the isolated population of *T. butleri* in southeastern Wisconsin.

The taxonomy of the *T. butleri* population in southeastern Wisconsin has been the subject of an ongoing debate for almost 100 years. As recently as 1981, it was believed that the range of *T. butleri* in southeastern Wisconsin covered 7 counties, from southern Fond Du Lac County south to the Illinois state line, and west to Walworth and Waukesha counties (Vogt 1981, see Figure 1). However, researchers have disagreed about the identity of many of the specimens upon which this range was based. Ruthven (1908) considered *T. butleri* and *T. radix* to be distinct species, but mentioned several specimens in southeastern Wisconsin that appeared to be intermediate. Davis (1932), however, concluded that specimens from Wisconsin were all true *T. butleri*, although more similar to *T. radix* than *T. butleri* from Michigan and Indiana. In 1949, Smith declared that *T. butleri* should be treated as a subspecies of *T. radix*, but Conant (1950) still considered *T. butleri* to be its own species. Casper (2003) conducted morphological analyses on *T. butleri*, *T. radix*, and suspected *T. butleri*-*T. radix* hybrids from several counties in

southeastern Wisconsin and found a geographic clustering of populations that is consistent with a clinal transition.

The taxonomic status of the two species is still unresolved across southeastern Wisconsin, although *T. butleri* meeting all the diagnostic criteria are probably limited to all or parts of only four counties: Milwaukee, Ozaukee, Washington, and Waukesha (Casper 2003). However, there are “good” *T. butleri* and “good” *T. radix*, based on morphology, 20 miles or so from where the two species come in contact. Thus, snakes that exhibit the morphological features that set these species apart live in the vicinity where the species overlap in southeastern Wisconsin. This study focuses on the behavior of neonatal *T. butleri* born to females originating from areas where the ranges of the species overlap (southeastern Wisconsin), as well as from areas outside the range of *T. radix* (Michigan and Ohio).

Hybridization and Conservation

Hybridization may play an important role in the evolution of both plants and animals (Allendorf et al. 2001). The evolutionary consequences of hybridization have received more attention from botanists than zoologists, although the same types of conservation problems arise in both plants and animals (Rhymer & Simberloff 1996). Within vertebrates, numerous cases of hybridization have been found among birds and mammals, with only a few among reptiles (Rhymer & Simberloff 1996). Although hybridization is sometimes beneficial, it can also be detrimental, because it can allow a more abundant species to drive a rare species into extinction (Levin 2002).

Morphological and genetic evidence support the possibility of hybridization of *T. butleri* and *T. radix*. Albright (2001) examined multiple paternity, morphology, and

behavior of *T. butleri* from northern Milwaukee county, located in southeastern Wisconsin. Although the litters used were from a “pure” *T. butleri* area, one litter in particular showed characteristics (both morphological and behavioral) of *T. radix*. Albright (2001) concluded that the similarity could have been because of “genetic swamping” between the two species.

Ford (1982) examined sex pheromone trails made by *T. butleri* and *T. radix*. *Thamnophis butleri* subjects were collected from lower Michigan and Ohio, and *T. radix* subjects were collected from central Illinois. In a Y-maze experiment, *T. butleri* males could not differentiate between their own female’s trails and the *T. radix* female’s trail. Although *T. butleri* subjects were collected from Michigan, these results still give evidence for some type of recent species separation.

Preliminary genetic data show that Wisconsin *T. butleri* may be a genetically distinct taxon that is either more recently derived from *T. radix* or has had more secondary contact with *T. radix* in post-glacial times than other *T. butleri* (Burghardt et al. unpubl. data). Burghardt and Casper (unpubl.) also concluded that, morphologically, there is a transition from “good” *T. butleri* to those containing *T. radix* characteristics in southeastern Wisconsin. Casper (2003) scored several morphological characters that are normally different between *T. butleri* and *T. radix*, and found consistent geographic clustering of species, with a probable zone of introgression. This study uses behavior as another way of answering this long-standing question in natricine snake systematics.

Behavior

Antipredator Behavior

How animals react when predators are present has important consequences for survival. In spite of their physical limitations, snakes have the most elaborate mechanisms for antipredator behavior yet described among reptiles (Greene 1988). Snakes elicit both active and passive defensive behaviors, including defecation, striking, death feigning, fleeing, tail rattling, and mimicry, among many others (Greene 1988). Mori and Burghardt (2004) proposed a change in terminology for describing antipredator behavior in snakes, stating that antipredator responses can be characterized by changes in distance between predators and prey, the amount of movement involved in the behavior, and in terms of their apparent function.

Antipredator behavior has been studied quantitatively in neonates in a variety of the species in the genus *Thamnophis*, beginning with Arnold and Bennett (1984) and Herzog and Burghardt (1986). Several types of antipredator behaviors have been described in this genus, including fleeing, striking, tail waving, body flattening, and head hiding. Past research has shown species differences in these reactions to predators (e.g., Herzog & Burghardt 1986). Bowers et al. (1993) compared the responses of several *Thamnophis* species to predatory threats, and found marked species differences in antipredator responses, attributing some of the differences to habitat and coloration differences. *Thamnophis butleri* rarely exhibited active antipredator behaviors other than escape, *T. marcianus* exhibited more striking behavior, but not as much fleeing as *T. butleri*, and *T. melanogaster* struck often (Bowers et al. 1993). *Thamnophis sauritus* fled and struck readily (Bowers et al. 1993).

Other factors are also involved. Recent feeding history and temperature can also have effects on the antipredator behavior of snakes (Herzog & Bailey 1987; Schieffelin & DeQueiroz 1991). Young *T. sirtalis* with full stomachs are more likely to strike at a threatening stimulus (Herzog & Bailey 1987), and adult and sub-adult *T. sirtalis* are more aggressive at higher temperatures (Schieffelin & DeQueiroz 1991). Brodie (1992) found a correlation between antipredator behavior and color pattern of *T. ordinoides*. The striped pattern of this garter snake allows it to become cryptic when it escapes from predators (Brodie 1992).

Past research on *T. butleri* and *T. radix* has shown differences in antipredator behavior. Bowers et al. (1993) found that, compared to the other species in a comparative study, *T. butleri* rarely exhibited any active antipredator behavior other than escape. Herzog and Burghardt (1986) found that the strikes of *T. butleri* newborns were less intense, often seeming hesitant, as compared to *T. sirtalis* and *T. melanogaster*. Herzog et al. (1992) found that *T. radix* from South Dakota and Michigan *T. butleri* showed similar low levels of antipredator responses other than flees, but these behaviors were only examined with non-contact moving and nonmoving visual stimuli. The snakes were not physically touched during the trials, which could have resulted in the lack of strong antipredator responses. The responses may have been different if a more severe stimulus was used in their study. However, in a study examining the antipredator displays of *T. radix*, Arnold and Bennett (1984) conducted tests by chasing each snake by prodding its tail with a cotton swab. A second trial was conducted by tapping each snake on the head, with some snakes being placed in a final trial where the snakes' tails were held. All snakes in these trials were chased to exhaustion before the antipredator

behaviors were measured. Arnold and Bennett (1984) found that more severe predatory attack (head-tapping and tail-holding) elicited more antipredator responses than milder attack. Thus, according to Arnold and Bennett's study, *T. radix* is more reactive when exposed to strong predatory attack.

Feeding Behavior

The examination of an organism's feeding behavior can yield information about what prey items the organism consumes, the types of foraging strategies used, and which types of prey items the organism seems to prefer. The wide variety of prey items consumed by *Thamnophis* species is one reason for the large amount of research on feeding behavior that has been conducted with these snakes.

Differences in the diet of *T. butleri* and *T. radix* have been documented.

Thamnophis butleri is an annelid specialist (Rossman et al. 1996). Carpenter (1952) conducted a large ecological study of Michigan gartersnakes and observed that *T. butleri* feed primarily on earthworms in the field, with leeches being the only other prey consumed. However, *T. butleri* would readily eat small minnows and chopped fish in the laboratory (Carpenter 1952). Burghardt and Hess (1968) also recorded captive animals of this species eating fish and even chopped horsemeat. *Thamnophis radix*, in contrast, is considered to be a generalist species, with a natural diet that can include earthworms, frogs, toads, fish, leeches, and mice, depending on location and season (Rossman et al. 1996).

Past research with gartersnakes has revealed differences in responses to prey stimuli due to ontogeny, geographical variation, past experience, and habitat. Snakes respond to chemical cues derived from species-typical prey with increased tongue-

flicking and even open-mouthed attack (Ford & Burghardt 1993). Burghardt examined the chemical preferences of several *Thamnophis* species, including *T. radix* and *T. butleri*. Both species performed more tongue-flicks to worm extracts than to fish extracts, but these differences were not significant (Burghardt 1967). Burghardt (1967) concluded that perhaps *T. butleri* has retained the potential to respond to chemical cues from fish, even though it does not consume this prey item in the wild. Burghardt also concluded that the stocky build of this species may render it incapable of capturing quick moving prey (Burghardt 1969).

Burghardt (1969) conducted a large comparative study of the chemically elicited prey attacks of *Thamnophis* species. *Thamnophis butleri* neonates responded to fish, amphibians, earthworms, and leeches, although twice as many attacks were made to the worm stimuli. *Thamnophis radix*, however, attacked the worm, fish, and leech extracts, but not the amphibian extract, with the attacks to worm stimuli being the highest (Burghardt 1969). Thus, perceptually, *T. butleri* was rather similar to the generalist *T. radix* in prey accepted in captivity and neonatal chemosensory preferences.

Lyman (1990) studied the development of chemosensory prey preferences in *T. butleri* from Michigan. Responses to the worm extract were significantly higher than responses to the fish extract. Of only nine attacks made to prey chemical stimuli, eight were made to worm.

Albright (2001) studied the behavior and morphology of neonates and adults from one population of *T. butleri* in Milwaukee County, WI. She found that the snakes exhibited an overall chemosensory preference for worms, but this preference was not significantly different from the chemosensory responses to fish. The snakes did show,

however, a significant preference for worms in prey preference tests (Albright 2001). Interestingly, some snakes consumed fish first in the prey preference tests, something never seen with *T. butleri* from Michigan.

Although *T. butleri* and *T. radix* have similar prey and chemosensory preferences, the two species do differ in the ability to capture prey. Drummond (1983) examined aquatic foraging in generalist and specialist gartersnakes, and found differences in their abilities to search for and capture prey items. Halloy and Burghardt (1990) examined developmental effects of fish capture experience in four species of *Thamnophis*, including both prey specialists (*T. butleri* and *T. melanogaster*) and prey generalists (*T. sirtalis* and *T. radix*). The two generalist species were less efficient in handling the fish than *T. melanogaster*, the aquatic specialist, but more efficient than *T. butleri*, the earthworm specialist (Halloy & Burghardt 1990).

Implications and Hypotheses

Thamnophis butleri and *T. radix* differ in several aspects of their behavior. *T. radix* are considered to be a more aggressive species compared to *T. butleri*. *Thamnophis butleri* are an annelid specialist and respond more to worm than fish extracts, while *T. radix* are a generalist species. Recent morphological and genetic data have revealed evidence of hybridization between these two species.

Several hypotheses have been put forth as to the interactions that occur when two species are sympatric. Character displacement can lead to the origin of novel phenotypes in response to competition among populations (Marko 2005). Based on this hypothesis, in my study, I would predict that (1) *Thamnophis butleri* in southeastern Wisconsin

would be less similar to *T. radix* than Michigan and Ohio *T. butleri*, which are found far outside the region of sympatry; (2) the behaviors of *T. butleri* from the different counties and populations in Wisconsin will differ from one another; (3) *Thamnophis butleri* from the southern part of their range in Wisconsin will show behaviors more like Michigan *T. butleri* and less like *T. radix* because of the close proximity of the two species. However, recent genetic and morphological evidence discount this hypothesis concerning *T. butleri* and *T. radix* in southeastern Wisconsin, so it will not be seriously considered as a viable hypothesis.

Another hypothesis emphasizes the importance of local ecology. In this hypothesis, it is the local ecology that drives the behaviors of the species examined. Based on this hypothesis, in my study, I would predict that (1) *T. butleri* in southeastern Wisconsin will be more similar to *T. radix* than Michigan and Ohio *T. butleri* because they share a similar habitat in southeastern Wisconsin; (2) the behaviors of *T. butleri* from the different counties and populations in Wisconsin will not differ from one another.

A final hypothesis concerning these species in southeastern Wisconsin is gene flow. Morphological and genetic evidence support the hypothesis of gene flow between *T. butleri* and *T. radix*, but this hypothesis has yet to be tested with only behavioral data. If my data support the hypothesis of hybridization in these species, I would predict that: (1) the behaviors of *T. butleri* in Wisconsin will differ from the behaviors of Michigan and Ohio *T. butleri* in the direction of being more like *T. radix*; (2) the behaviors of *T. butleri* from the different counties and populations in Wisconsin will differ from one another; (3) *Thamnophis butleri* from the northern part of the Wisconsin range, further removed from the range of *T. radix*, will show behaviors more like Michigan *T. butleri*

and less like *T. radix* than do *T. butleri* from the southern part of their range in Wisconsin. The behavioral data will be compared, in a preliminary way, with existing morphological and genetic evidence for the hybridization of these two species in Wisconsin.

2. METHODS

General Methods

Adult Females

Pregnant *T. butleri* (n= 16) and *T. radix* (n= 2) were brought into the Reptile Ethology laboratory at the University of Tennessee, Knoxville in May and July, 2004. *Thamnophis butleri* were captured in Wisconsin, Michigan, and Ohio. The pregnant females from Wisconsin were collected from 4 counties: Milwaukee, Ozaukee, Racine, and Waukesha (see Figure 2 for map of collection locations in Wisconsin and Tables 1 and 2 for distances between the populations). The pregnant females from Michigan (Sterling State Park) and Ohio (Toledo) were used for comparative purposes. Pregnant female *T. radix* were captured in Will county, IL (Gooderow Grove Nature Preserve). It was difficult to locate *T. radix* in this area and, as a result, the number of subjects is lower than desired. Also, another pregnant *T. radix* was captured, but all offspring were stillborn. The adult females were caught by Tom Anton, Kent Becker, Gordon Burghardt, and Gary Casper. Pregnant females from both species were individually housed in clear, plastic cages (405 mm x 260 mm x 155 mm) lined with commercial cage liner made from corrugated cardboard (Shepherd Specialty Papers). A hide box and water bowl were present in each cage. A heat strip was placed under half of the cage to allow for thermoregulation. Females were fed a diet of night crawlers, *Lumbricus terrestris*, or mice, *Mus musculus*, twice per week. Water was available *ad libitum*.

Offspring

The test subjects were neonate *Thamnophis butleri* (n=160) and *T. radix* (n=12) born in the Reptile Ethology Laboratory at the University of Tennessee to the 18 wild-

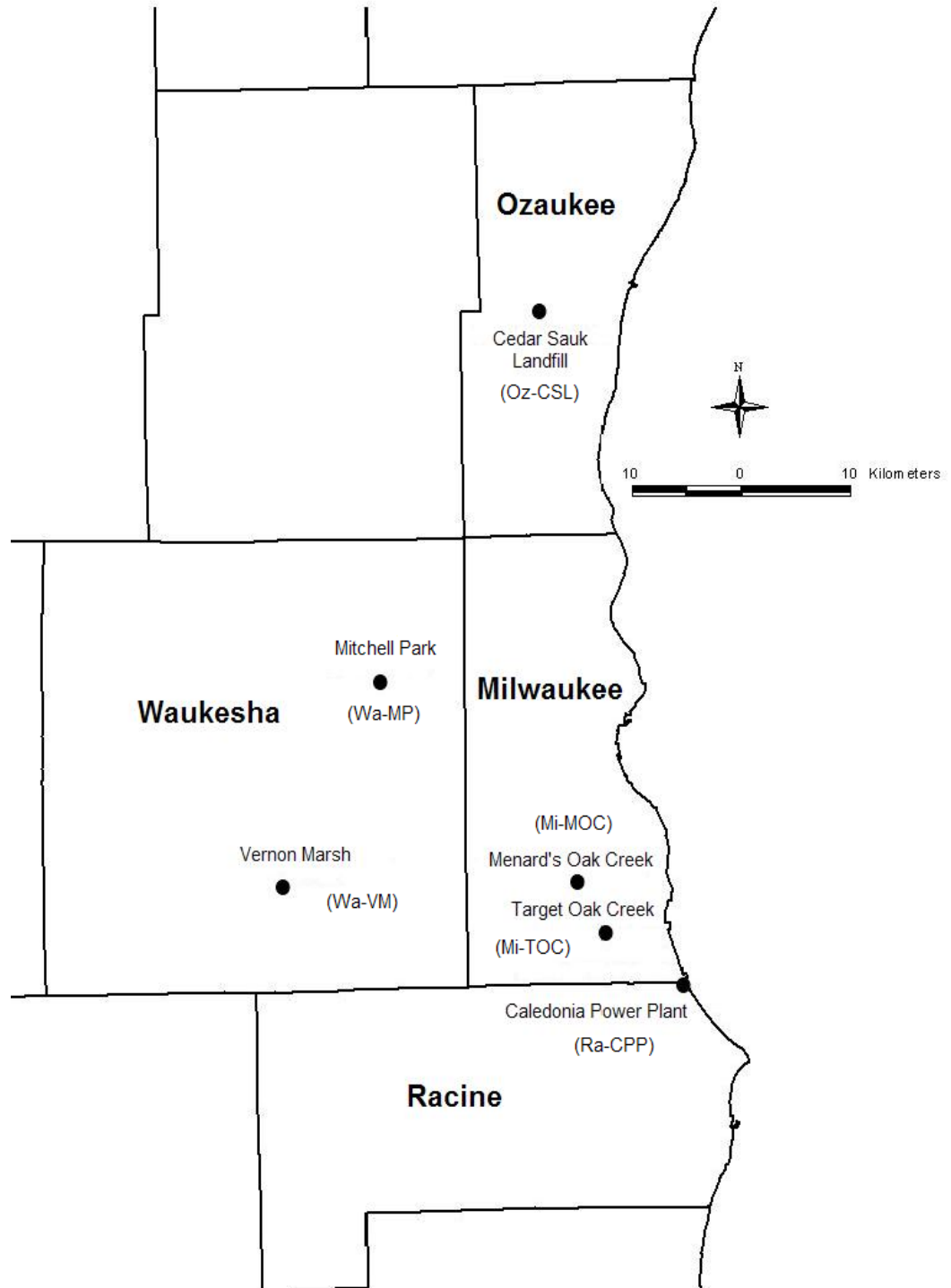


Figure 2. Collection sites of *Thamnophis butleri* from four counties in southeastern Wisconsin.

Table 1. Distance measurements (km) between populations in southeastern Wisconsin. See Fig. 1 for identity of acronyms identifying populations.

	Wa-MP	Wa-VM	Mi-MOC	Mi- TOC	Ra-CPP
Oz-CSL	34.8	53.9	48.7	53.0	58.7
Wa-MP		19.8	24.8	29.1	37.8
Wa-VM			26.5	29.1	37.0
Mi-MOC				5.2	13.0
Mi-TOC					8.3

Table 2. Distance measurements (km) between populations of *Thamnophis* in Ohio, Illinois, and Michigan from the most southeastern Wisconsin population.

	Will (IL)	Toledo (OH)	Sterling (MI)
Ra-CPP (WI)	203	515	562
Will (IL)		425	462
Toledo (OH)			50

caught females (see Table 3). Neonate snakes were used in this study to eliminate the effects of habituation and other types of prior experience.

All neonates were born between July 1 and August 21, 2004. Immediately after birth, all snakes were weighed, measured, and separated. Snakes were individually housed in clear, plastic boxes (180 mm x 140 mm x 55 mm) lined with corrugated cardboard. A water dish and paper hide box were present in each box. Water was available *ad libitum*. Temperature was maintained at approximately 20° C and lighting was on a 12:12-hr. light-dark cycle.

After completion of all behavioral trials, the snakes were fed a diet of night crawlers, *Lumbricus terrestris*, twice per week. Neonates were sexed by hemipenal eversion after completion of all behavioral trials.

Morphology

Adult snakes were weighed and measured upon arrival into the laboratory and again post-partum. Neonate snakes were measured immediately after birth and again between October 28 and November 23, 2004 in order to assess growth in mass and length. Any subjects that died were weighed and measured at time of death.

Masses of snakes to nearest 0.01g were taken using a digital scale. Length measurements, including snout-vent length (SVL) and tail length (TL), were measured to nearest 1.0 mm with a meter stick. A body condition index that is unbiased for size was calculated for adult females and neonates by taking the cube root of the mass and dividing it by the SVL (Rivas 2000). I multiplied this number by ten to reduce the number of zeros. Comparisons of litters and populations were made only among neonatal individuals and in comparing neonates with their mother. This index assumes that larger

Table 3. Litters born to *Thamnophis butleri* and *T. radix* females from Illinois, Michigan, Ohio, and Wisconsin (actual subjects tested).

Species	State	County	Site	Litter #	DOB	n	Sex ratio (M/F)
<i>T. butleri</i>	MI	Monroe	SSP	1	7/1/04	9	4/5
<i>T. butleri</i>	MI	Monroe	SSP	2	7/11/04	9	3/6
<i>T. butleri</i>	OH	Lucas	Toledo	10	7/22/04	16	8/8
<i>T. butleri</i>	WI	Milwaukee	TOC	7	8/6/04	10	4/6
<i>T. butleri</i>	WI	Milwaukee	TOC	8	8/5/04	8	6/2
<i>T. butleri</i>	WI	Milwaukee	MOC	6	7/29/04	13	5/8
<i>T. butleri</i>	WI	Ozaukee	CSL	22	8/11/04	8	4/4
<i>T. butleri</i>	WI	Ozaukee	CSL	25	8/10/04	4	0/4
<i>T. butleri</i>	WI	Ozaukee	CSL	27	8/12/04	11	5/6
<i>T. butleri</i>	WI	Racine	CPP	14	8/6/04	14	5/9
<i>T. butleri</i>	WI	Racine	CPP	17	8/3/04	8	4/4
<i>T. butleri</i>	WI	Racine	CPP	18	8/9/04	10	6/4
<i>T. butleri</i>	WI	Racine	CPP	19	8/13/04	8	5/3
<i>T. butleri</i>	WI	Waukesha	VM	20	8/18/04	15	6/9
<i>T. butleri</i>	WI	Waukesha	VM	21	8/5/04	10	7/3
<i>T. butleri</i>	WI	Waukesha	MP	5	8/8/04	8	4/4
<i>T. radix</i>	IL	Will	GGNP	12	8/16/04	2	1/1
<i>T. radix</i>	IL	Will	GGNP	13	8/21/04	10	6/4

animals have the same shape as smaller animals, which was not an assumption of previous condition indexes (Rivas 2000).

Antipredator Behavior

Antipredator tests were conducted 2 days after each snake was born. Prior to testing, individuals were brought into the testing room for at least 20 min. After this time, snakes were placed in a glass aquarium (605 mm x 528 mm x 255 mm) with an artificial turf substrate. The sides of the aquarium were lined with cardboard, to prevent the snake from seeing its reflection and any outside objects. A Graylab 451 timer was used to time trials. The testing procedure was adapted from Bowers et al. (1993).

Snakes were placed in the aquarium and, after a 30 s undisturbed period, were subjected to three levels of predatory threat, in order of increasing threat level. Each threat level lasted for 1 min. with a 30 s undisturbed period between each level. In Level 1, a nonmoving index finger was placed 1-2 cm from the snake's snout. During Level 2, an index finger was again placed 1-2 cm from the snake's snout, but this time the finger moved at a rate of 2-3 oscillations per second. During the final stage of the trial, Level 3, the index finger tapped the head of the snake at a rate of approximately once per second. The frequency of six behaviors was recorded during each of the three levels: striking, biting, fleeing, body or head flattening, tail wagging, and head hiding (see Table 4 for descriptions of behaviors). The substrate was wiped with alcohol and the experimenter's hands were washed between subject trials. Trials were conducted between 800-1300 hours. Temperatures ranged from 23-24 °C. Snakes were tested for their antipredator responses again 22-25 days after birth, in order to test for ontogenetic shifts in the behavior, as well as to assess the presence of habituation.

Table 4. Description of antipredator behaviors exhibited by *Thamnophis butleri* and *T. radix*.

Behavior	Explanation
Striking	The snake lunges in the general direction of the index finger with or without an open mouth.
Biting	Occurs with a strike, but the snake's mouth latches onto the experimenter's finger.
Fleeing	The snake moves to another part of the aquarium, in a direction opposite to the experimenter's finger.
Head or Body Flattening	The snake flattens its head or anterior portion of its body- usually occurs prior to a strike or bite.
Tail Waving	The snake's tail oscillates from one side to the other, usually raised from the substrate.
Head Hiding	The snake's head is hidden under its tail or body.

Chemosensory Tests

Chemosensory tests were conducted 9 or 10 days after birth, without any previous encounter with prey items. Twenty-four hours prior to the tests, snakes were brought into the testing room and individually placed in plastic cages (155 mm x 130 mm x 90 mm), with only a substrate of corrugated cardboard. Tests were conducted in cages with higher sides than their home boxes to reduce escapes. The sides of the testing containers were covered with cardboard to eliminate visual contact between snakes.

Stimuli consisted of prey extracts (fathead minnows, *Pimephales promelus*, and earthworms, *Lumbricus terrestris*) and deionized water (control). Two litters were also tested with a mouse extract, *Mus musculus*, as part of another experiment. Responses to the mouse extract were deleted for the purposes of this study. Aqueous surface prey extracts were prepared with procedures used for many years (Burghardt 1993). Prey items were patted dry and weighed. The ratio used was 3 g of prey to 10 cc of deionized water. The prey was placed in the measured amount of deionized water and heated to 60° C for 2 min. The liquid was then poured off and centrifuged for 10 min. The supernatant was then poured into vials and frozen. The prey extracts were thawed the morning of chemosensory trials.

Each snake was tested twice with each of the three different stimuli. The extracts and water control were presented for 30 s in a systematic order, followed by the presentation of the stimuli in reversed order to control for order effects. There were six possible orders of the three stimuli, which entailed that each stimulus was presented the same number of times, and that the stimuli were presented in a systematically balanced order. A period of at least 20 min. occurred between each test. The number of total

tongue-flicks and tongue-flicks to the stimuli were recorded, as well as the number of attacks. If the snake attacked the stimulus, the trial ended and the latency of the attack was recorded.

The tongue-flick and attack data were combined into a tongue-flick attack score (TFAS) (Cooper & Burghardt 1990). This measure incorporates both tongue-flicks and latency to attack into a single index. TFAS(R) for repeated measures was calculated by adding the greatest number of tongue-flicks for any stimulus, given by each individual in any trial without an attack, to the latency component. The equation is as follows:

$$\text{TFAS(R)}_i = \text{TF}_{\max(i)} + (\text{TL} - \text{latency}_i)$$

where $\text{TF}_{\max(i)}$ is the maximum number of tongue-flicks emitted by individual i in any trial, TL is trial length in seconds in the absence of an attack, and latency_i is the latency of attack by an individual i .

The TFAS(R) was natural log transformed (score+1) in order to reduce the variance and heteroscedasticity. Throughout the rest of the paper, TFAS will be used when referring to the tongue-flick attack score.

Prey Preference Test

Prey preference tests were conducted 14-17 days after birth. All trials were conducted in the snake's home cage. Prior to the introduction of the prey items, the water dish and paper hide box were removed. Two petri dishes were then placed simultaneously in the snake's cage, one containing fish (F), *Poelicia reticulata*, and the other containing a piece of leafworm (W), *Lumbricus rubellis*. The sizes of the two prey items were approximately equal. Both species of prey items were different than those

used in chemosensory testing because of the size of the prey items. The prey items used in chemosensory testing were too large for neonates to consume.

During the first presentation of food items, the fish was placed in about 5 mm of water and the worm was placed in the same amount of soil. The snakes were observed continuously for one hour for ingestion of food. If the snake consumed a prey item during this time period, the prey item that was consumed was recorded, as well as the latency to consume the prey item. The snakes were observed again after 2 and 24 hours in order to assess if the snake had consumed the other prey item.

If the snake did not eat either of the prey items after one hour, the prey items were removed and the test was conducted in the same fashion 3 days later. If the snake did not eat a second time, a third trial was conducted in which only the prey items were presented in the petri dish (no water or dirt). The prey preference tests were continued until all snakes chose at least one prey item, or until a total of 5 prey preference trials were conducted, whichever came first.

For the prey preference tests, I recorded which prey item was consumed first, and the latency of consumption of the prey item. I also recorded whether or not the snake consumed the other prey item and the latency to consume the second item.

Statistical Analyses

General

Sex, species, population, and litter differences were compared using an analysis of variance in all morphological and behavioral measures. Population was specified as a fixed factor and litter was treated as a random factor nested within population. I also performed an analysis of variance in order to investigate differences between the

behaviors of *T. butleri* from southeastern Wisconsin and the other *T. butleri* from Ohio and Michigan, as well as to compare the measures among the populations of southeastern Wisconsin. Where multiple comparisons were conducted, Holm's method was used for correcting for significance of p-values (Aickin 1996). All data analyses were conducted with SPSS 12.0 software and graphs were prepared with SPSS 13.0.

Also, comparisons between the most northern (Ozaukee county) and most southern (Racine county) populations are emphasized because these were from the most pure *T. butleri* and most *T. radix*-impacted populations, respectively. These comparisons were the most critical for testing the hybridization hypothesis. Also, the most litters were from these populations.

Morphology

Neonate SVL, mass, and TL were compared using an ANOVA. In the last two measures, SVL was used as a covariate, as well as alone. These comparisons will allow me to reveal both species, as well as population, differences in order to test the hypotheses of this study. I used a simple linear regression to test for correlations between maternal and offspring SVL and mass. I also tested for correlations between maternal mass and number of offspring, as well as correlations between maternal SVL and number of offspring.

Growth comparisons were tested with a paired t-test. In order to assess differences in growth between populations, a growth variable was created by subtracting the first measurements from the second measurements and then dividing this number by the number of days between the two measurements. This growth variable was compared between populations using an analysis of variance.

Antipredator Behavior

Antipredator behavior data were natural log transformed (score + 1) in order to reduce the variance and heteroscedasticity. To assess differences in behavior of the snakes from southeastern Wisconsin, an analysis of variance was performed for striking, fleeing, flattening of the head or body, and tail waving. Bites were added to the number of strikes, because of the low occurrence of biting. In order to assess differences in the presence or absence of antipredator behaviors, I also conducted a chi-squared test for the flattening and tail waving behaviors. I performed the analyses for both the first and second antipredator tests (Day 2 and Day 22-25, respectively).

I performed a repeated measures ANOVA to assess differences in antipredator responses of striking and fleeing between the threat levels (still, moving, touching). Differences between the first and second antipredator tests were compared using paired t-tests.

Chemosensory Tests

Responses to the control, worm, and fish stimuli were compared using a repeated measures analysis of variance. I conducted this test for the first and second presentations of the stimuli and for the average of the two presentations. I tested for an order effect of the stimuli with a repeated measures analysis of variance. I also tested for the direction of the chemosensory preference by subtracting the fish TFAS from the worm TFAS for each individual (W-F). This was compared between all populations in southeastern Wisconsin using an analysis of variance. I also compared the number of attacks to each stimulus using a chi-squared test, and compared the number of attacks among populations.

Correlations among Behavioral and Morphological Measures

After analyzing the behavioral and morphological results, I used the measures from each test that best discriminated among populations to test for correlations. I combined morphological measurements (mass, SVL, body condition index), antipredator behaviors (strikes, flees), and feeding behaviors (attacks to stimuli, TFAS to water, fish, and worm) in order to test for correlations among these variables, using a Pearson Correlation test.

Discriminant Function Analysis

I also performed a discriminant function analysis (DFA) to identify the variables that best distinguished between populations. I first ran the DFA using all Wisconsin populations, and then using only Ozaukee vs. Racine and Milwaukee vs. Waukesha animals. I used only behavioral data for these analyses, including antipredator (strikes and flees) and chemosensory (TFAS worm and fish, and W-F TFAS) behaviors.

3. RESULTS

In general, neonatal sex differences were small and statistically insignificant. They will be mentioned as appropriate and selected results are in the Appendix. Litter differences within populations were often significant and the details of these differences will also be found in the Appendix. The results that follow primarily present details of the species and population differences that are the main focus of this study.

In comparing populations, the Michigan and Ohio *T. butleri* will sometimes be pooled as MI/OH. The Wisconsin *T. butleri* may often be pooled together, and the Racine and Ozaukee populations will often be compared with each other, as they are the key populations for testing clinal differences, being the most northern and southern populations, respectively. Also, the largest numbers of litters were from these two populations. The four intermediate populations in Milwaukee and Waukesha counties will sometimes be referred to collectively as Mil/Wau. Population comparisons using all neonates as well as neonates nested in litters within populations will be presented at times.

Morphology

Snout-Vent Length

Thamnophis radix neonates had significantly longer SVLs than all *T. butleri* ($F_{1,171} = 91.40$, $p = 0.000$, Figure 3). *Thamnophis radix* neonates also had significantly longer SVLs than Michigan and Ohio *T. butleri* combined ($F_{1,46} = 39.96$, $p = 0.000$), as well as all Wisconsin *T. butleri* combined ($F_{1,137} = 100.37$, $p = 0.000$). However, *T. butleri* neonates from MI/OH had significantly longer SVLs than snakes from Wisconsin ($F_{1,159} = 9.06$, $p = 0.003$).

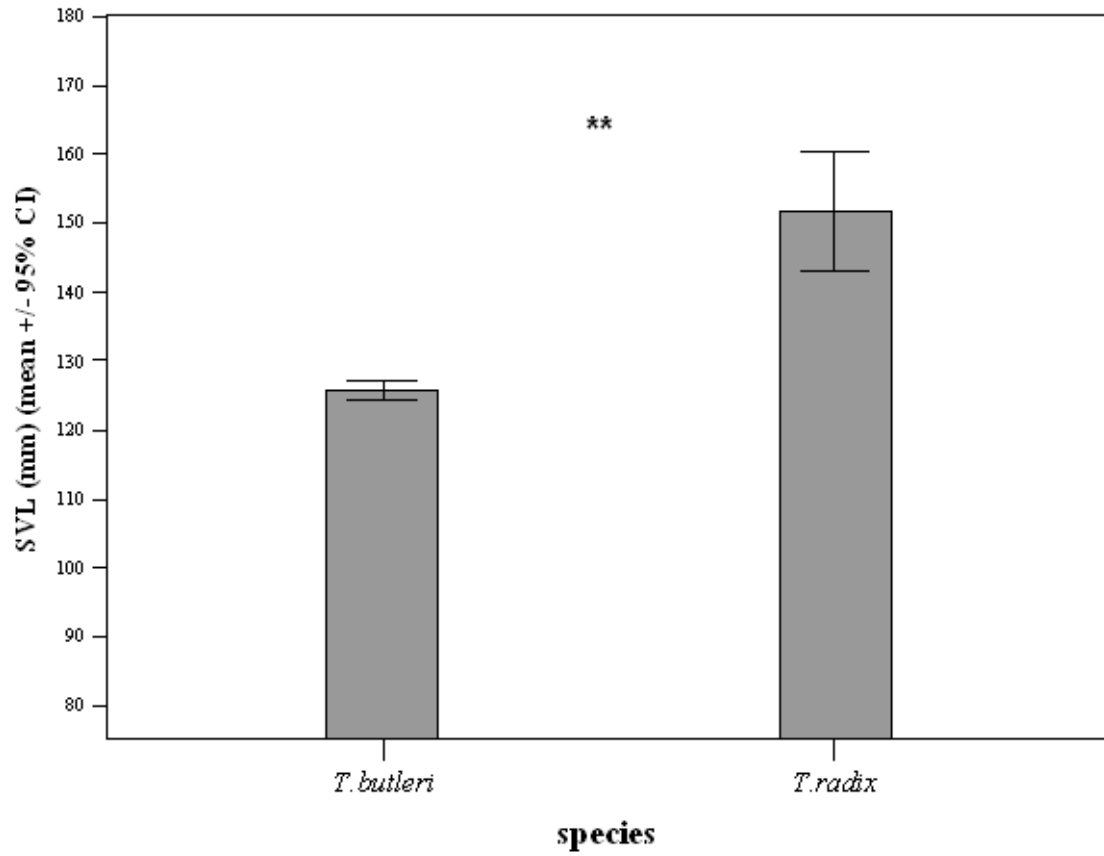


Figure 3. Offspring snout-vent length of *Thamnophis butleri* and *T. radix*.
** = $p \leq 0.01$

Significant population effects, with litter nested within population, were found among the populations of southeastern Wisconsin for SVL ($F_{5, 7} = 7.24$, $p = 0.000$, Figure 4). Snakes from Racine were significantly longer in SVL than snakes from Ozaukee ($p = 0.002$). See Table 5 for results of all population differences.

Mass

Thamnophis radix subjects weighed more than combined *T. butleri* subjects ($F_{1, 171} = 33.86$, $p = 0.000$, Figure 5). However, *T. butleri* subjects weighed more than *T. radix* snakes ($F_{1, 171} = 7.20$, $p = 0.008$) when mass was examined with SVL as a covariate.

Thamnophis radix snakes weighed significantly more than MI/OH *T. butleri* ($F_{1, 46} = 9.97$, $p = 0.003$), but the relationship was reversed when mass was examined with SVL as a covariate ($F_{1, 46} = 5.44$, $p = 0.025$). *Thamnophis radix* snakes weighed more than Wisconsin *T. butleri* ($F_{1, 137} = 45.70$, $p = 0.000$), but when mass was examined with SVL as a covariate, Wisconsin *T. butleri* subjects weighed significantly more than *T. radix* ($F_{1, 137} = 3.85$, $p = 0.052$).

MI/OH *T. butleri* weighed significantly more than Wisconsin *T. butleri* both when mass was examined with SVL as a covariate ($F_{1, 159} = 14.59$, $p = 0.000$) and when mass was examined without SVL as a covariate ($F_{1, 159} = 22.99$, $p = 0.000$).

Significant population effects were found among the populations of southeastern Wisconsin for mass ($F_{5, 7} = 6.90$, $p = 0.000$, Figure 6) when mass was examined without SVL as a covariate. Snakes from Racine weighed significantly more than snakes from Ozaukee ($p = 0.008$). See Table 5 for results of all population differences.

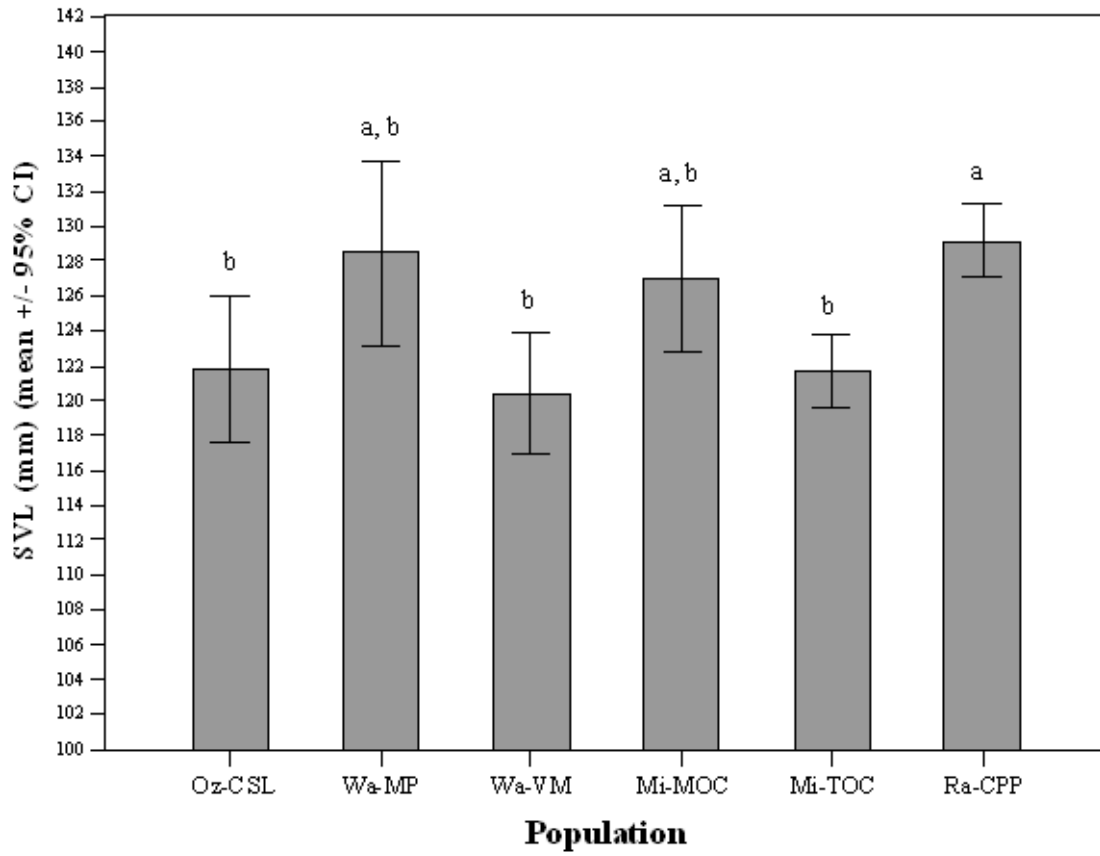


Figure 4. Lengths (SVL) of southeastern Wisconsin populations of *Thamnophis butleri*. Columns labeled with different letters are significantly different ($p \leq 0.05$).

Table 5. Statistical differences among Wisconsin *Thamnophis butleri* populations for snout-vent length and mass. All values in bold face are statistically different ($p \leq 0.05$) after Holm's correction.

		Wa- MP	Wa- VM	Mi- MOC	Mi- TOC	Ra- CPP
Oz- CSL	SVL mass	0.223 0.310	0.989 0.419	0.330 0.267	1.000 0.297	0.002 0.008
Wa- MP	SVL mass		0.075 0.010	0.997 1.000	0.236 0.998	0.999 1.000
Wa- VM	SVL mass			0.101 0.003	0.994 0.002	0.000 0.000
Mi- MOC	SVL mass				0.352 1.000	0.883 0.993
Mi- TOC	SVL mass					0.003 0.936

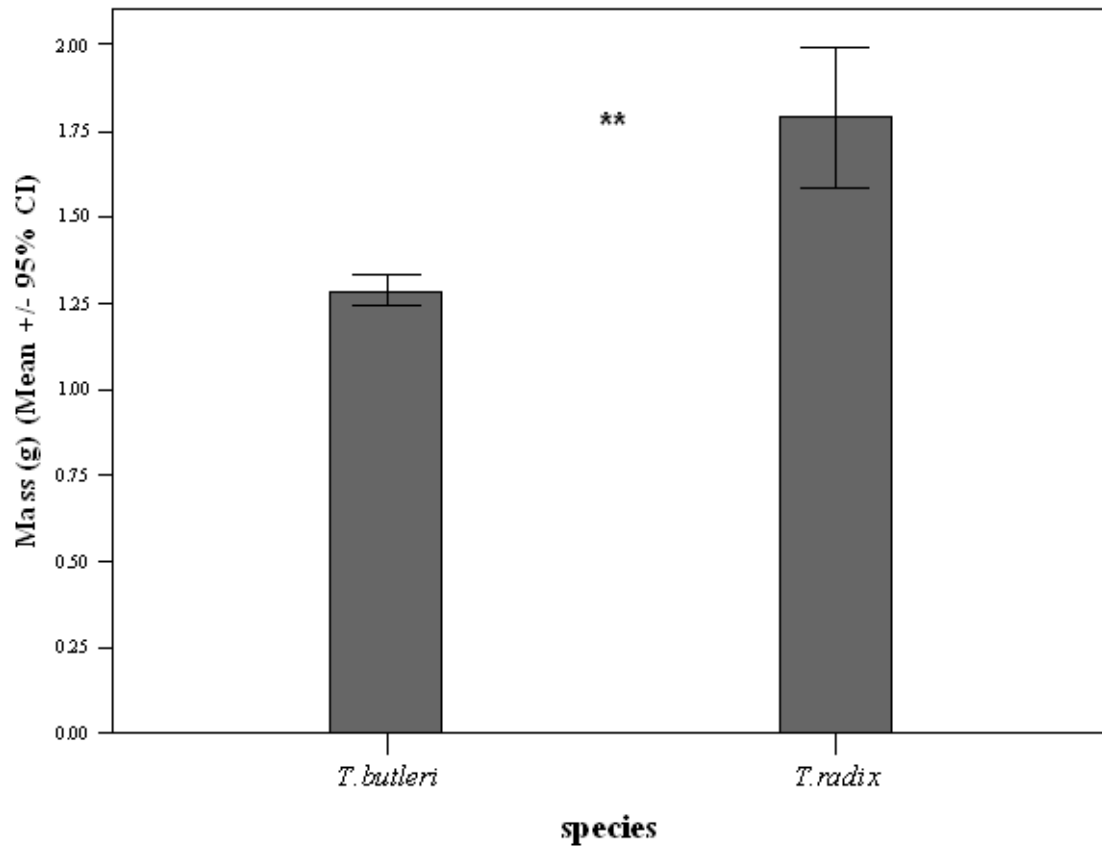


Figure 5. Comparison of mass of *Thamnophis butleri* and *T. radix*. Significant species effects were found across all snakes.
** = $p < 0.01$

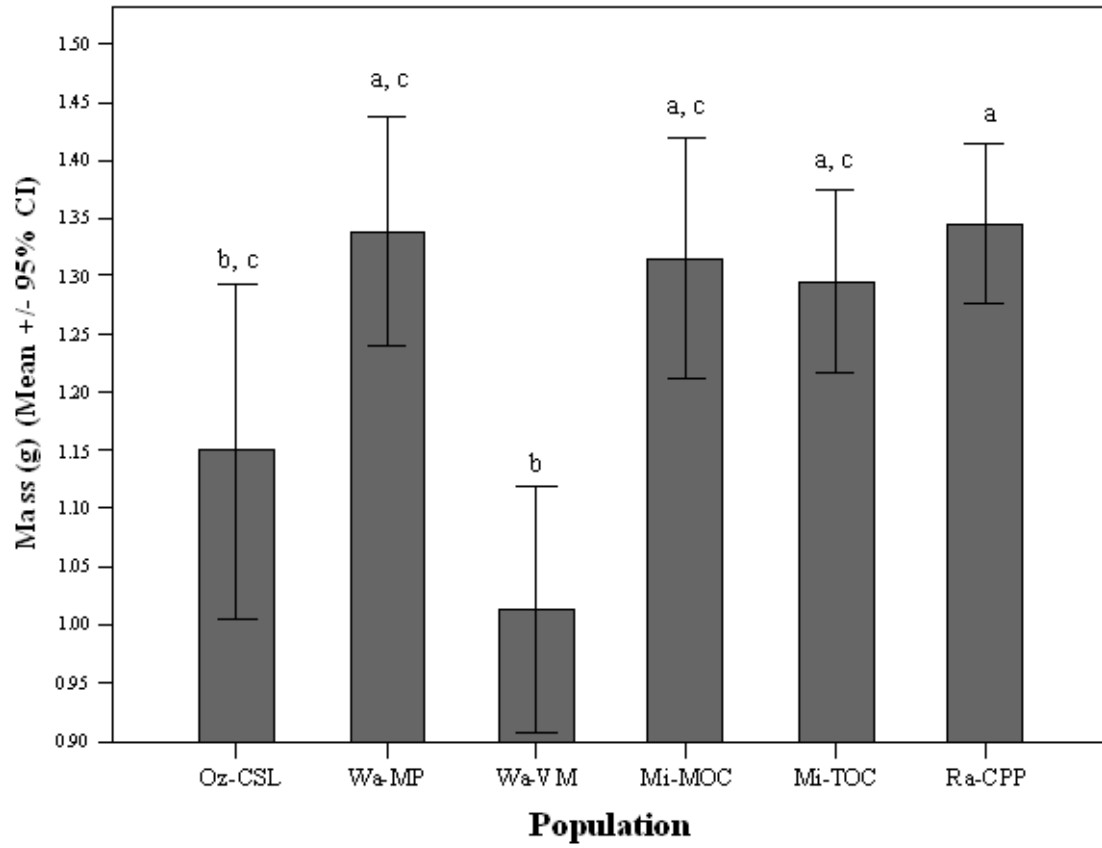


Figure 6. Comparisons of mass of populations of *Thamnophis butleri* from southeastern Wisconsin. A significant population effect was found. Columns labeled with different letters are significantly different ($p \leq 0.05$).

Body Condition

There was a significant difference between the species in the body condition index ($F_{1,172} = 25.96$, $p = 0.000$), with *T. butleri* snakes having a higher condition index than *T. radix* snakes, documenting that *T. butleri* are the more stocky species. MI/OH *T. butleri* had a significantly higher body condition index than *T. radix* subjects ($F_{1,45} = 36.85$, $p = 0.000$), as did Wisconsin *T. butleri* ($F_{1,138} = 24.81$, $p = 0.000$). However, MI/OH *T. butleri* had a significantly higher body condition index than Wisconsin *T. butleri* ($F_{1,160} = 8.29$, $p = 0.005$), suggesting that Wisconsin *T. butleri* are closer to Illinois *T. radix* in body shape.

There was a significant population effect between the populations from southeastern Wisconsin in condition index ($F_{5,126} = 8.27$, $df = 5$, $p = 0.000$). Although Ozaukee and Racine animals did not differ, snakes from Milwaukee-Target Oak Creek had a significantly higher body condition index than snakes from Ozaukee ($p = 0.002$), Racine ($p = 0.000$), and Waukesha-Vernon Marsh ($p = 0.000$). See Figure 7 for all population comparisons.

Growth

Growth rate measures were found to be strongly influenced by a snake's willingness to consume prey items, and because this confounded the population comparisons, results are found in the Appendix.

Maternal and Offspring Correlations

Simple linear regressions showed a significant correlation between pre-partum mass and the total number of offspring (see Table 6) and between pre-partum mass and the number of live offspring. The total number of offspring included all offspring born,

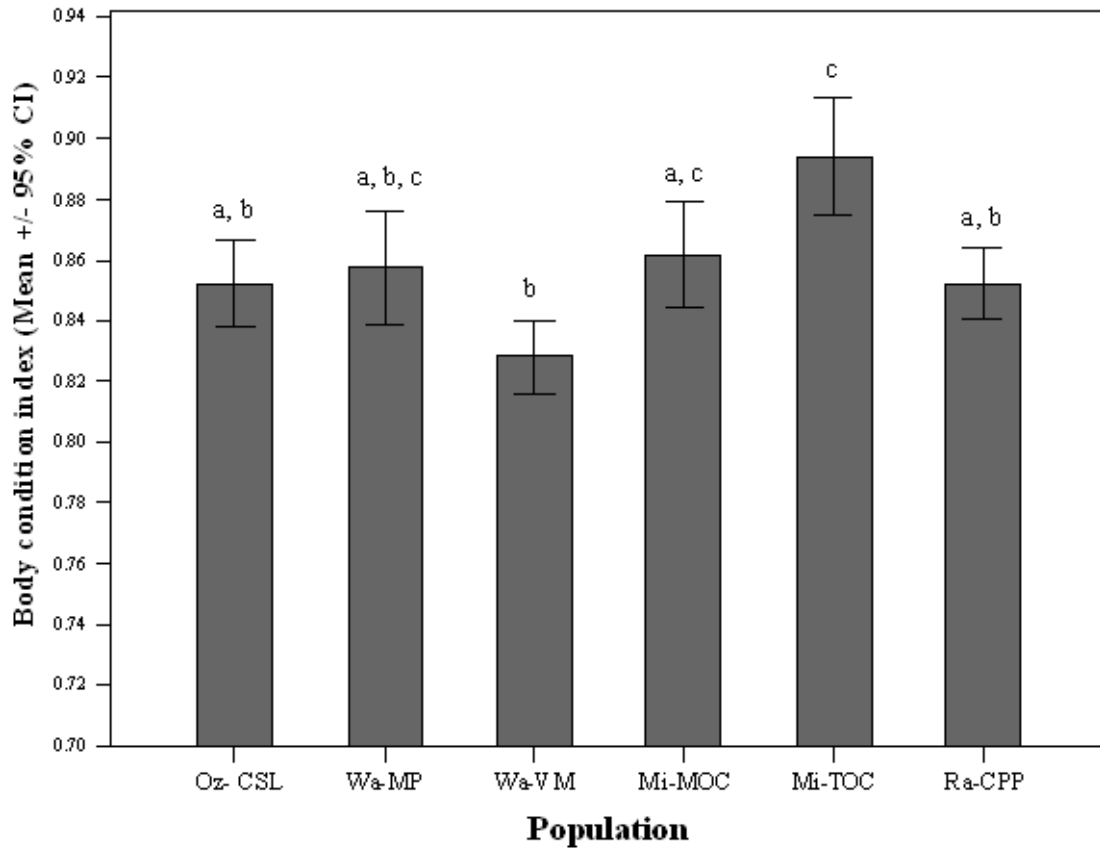


Figure 7. Comparisons of the body condition index of *Thamnophis butleri* populations from southeastern Wisconsin. A significant difference was found among the populations. The body condition index was derived from the cube root of the individual's mass divided by the individual's SVL (as explained in the text). Columns labeled with different letters are significantly different ($p \leq 0.05$).

Table 6. Results of linear regression examining maternal/offspring correlations of all individuals of *Thamnophis butleri*.

	r	R ²	F	df	p
Pre-partum maternal mass/ total no. offspring	0.701	0.492	13.54	1,16	0.002
Pre-partum maternal mass/ no. live offspring	0.669	0.447	11.32	1,16	0.005
Pre-partum maternal mass/ mean offspring mass	0.144	0.021	0.30	1,16	0.594
Pre-partum maternal mass/ mean offspring SVL	0.038	0.001	0.02	1,16	0.890
Maternal SVL/ total no. offspring	0.755	0.570	18.54	1,16	0.001
Maternal SVL/ no. live offspring	0.532	0.283	5.53	1,16	0.034*
Maternal SVL/mean offspring mass	0.210	0.044	0.65	1,16	0.435
Maternal SVL/mean offspring SVL	0.214	0.046	0.67	1,16	0.426
Pre-partum maternal body condition/ mean offspring body condition	0.362	0.131	2.11	1,16	0.168
Pre-partum maternal body condition/ total no. offspring	0.006	0.000	0.00	1,16	0.984
Pre-partum maternal body condition/ no. live offspring	0.326	0.106	1.67	1,16	0.218

* p-value not significant with Holm's correction

even those born as stillborns. Significant correlations were found between maternal SVL and number of live offspring and between maternal SVL and total number of offspring. There was no association of maternal mass or SVL with offspring mass or SVL. No significant correlations were found between pre-partum maternal body condition and offspring body condition, or between pre-partum maternal body condition and number of offspring (total or live).

Antipredator Behavior

Here I present the results from the initial (Day 2) and the second (Day 22-25) antipredator test for all the snakes. Tail waves and head/body flattening were low frequency and best characterized as present or absent in individuals. I thus used chi-squared tests to examine differences in the presence or absence of these behaviors, in addition to an analysis of number of responses by individuals. Results from the ANOVA tests are presented first, followed by results from the chi-squared tests.

Initial Test

Thamnophis radix were more likely to strike than were *T. butleri* ($F_{1,172} = 7.42$, $p = 0.007$, see Figure 8), whereas *T. butleri* were more likely to flee than were *T. radix* ($F_{1,172} = 8.65$, $p = 0.004$, see Figure 9). No significant species differences were found for the amount of flattening ($F_{1,172} = 0.00$, $p = 0.974$) or tail waving ($F_{1,172} = 2.72$, $p = 0.101$).

Thamnophis radix subjects were significantly more likely to strike than were MI/OH *T. butleri* ($F_{1,46} = 25.85$, $p = 0.000$), while MI/OH *T. butleri* were more likely to flee ($F_{1,46} = 33.28$, $p = 0.000$) and tail wave ($F_{1,46} = 6.70$, $p = 0.013$) than were *T. radix*. No significant species differences were found for flattening ($F_{1,46} = 0.22$, $p = 0.641$).

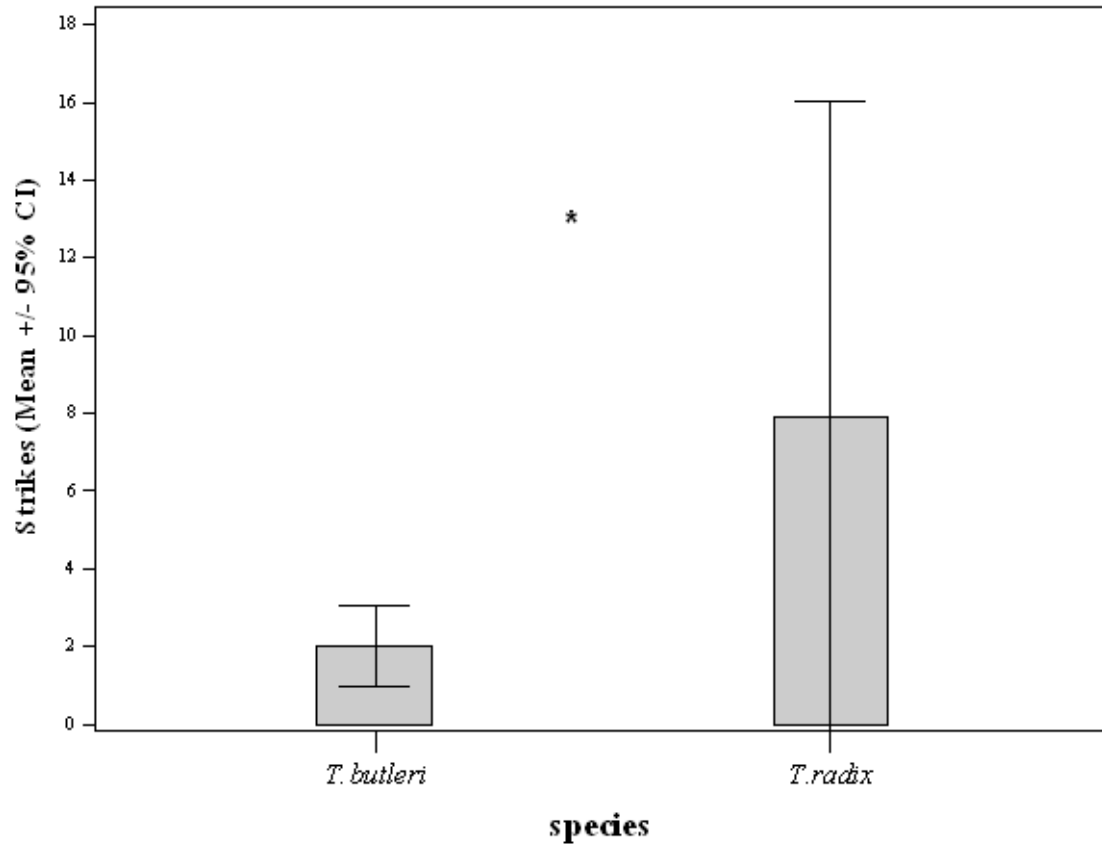


Figure 8. Comparisons of strikes of *Thamnophis butleri* and *T. radix* two days post-partum. A significant species effect was found.
* = $p \leq 0.05$

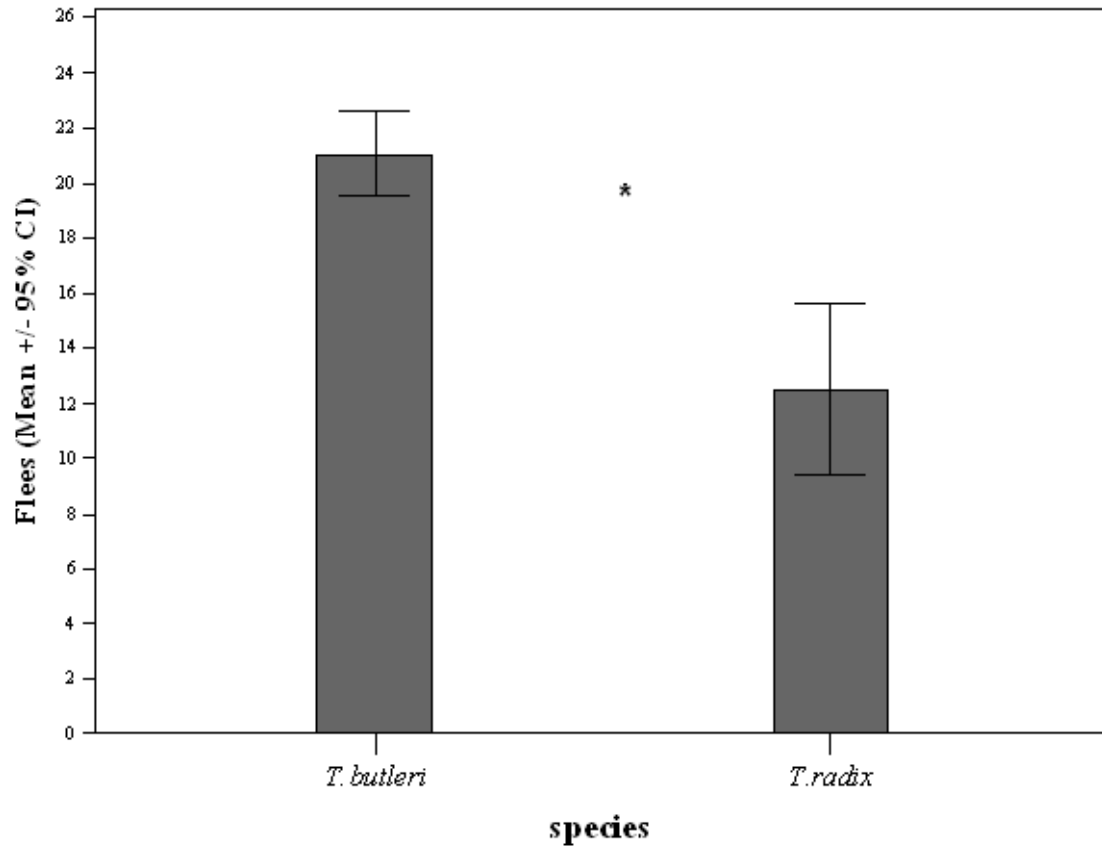


Figure 9. Comparisons of flees of *Thamnophis butleri* and *T. radix* two days post-partum. A significant species effect was found.
* = $p \leq 0.05$

Compared to Wisconsin *T. butleri*, *T. radix* subjects were more likely to strike ($F_{1, 137} = 8.80$, $p = 0.004$) but less likely to flee ($F_{1, 137} = 5.07$, $p = 0.026$). No significant differences were found in flattening ($F_{1, 137} = 0.00$, $p = 0.975$) or tail waving ($F_{1, 137} = 1.71$, $p = 0.193$).

MI/OH *T. butleri* were more likely to flee ($F_{1, 159} = 46.91$, $p = 0.000$) and tail wave ($F_{1, 159} = 12.95$, $p = 0.000$) than *T. butleri* from Wisconsin. No significant differences were found in striking ($F_{1, 159} = 2.01$, $p = 0.159$) or flattening ($F_{1, 159} = 0.24$, $p = 0.625$).

Neither strikes ($F_{5, 7} = 0.85$, $p = 0.557$), flees ($F_{5, 7} = 0.40$, $p = 0.838$), flattenings ($F_{5, 7} = 1.90$, $p = 0.220$), or tail waves ($F_{5, 7} = 0.53$, $p = 0.751$) differed among the populations of southeastern Wisconsin when litter was nested within population (see Figures 10, 11).

Snakes from Racine were more likely to strike than snakes from any of the other populations in southeastern Wisconsin, although these differences were not significant in the nested analysis. Snakes from Racine were also more likely to exhibit head or body flattening than snakes from the other populations, but again, these differences did not reach statistical significance.

When population differences were examined without litter nested within population, significant differences were found in the number of strikes ($F_{5, 126} = 2.45$, $p = 0.038$) and flees ($F_{5, 126} = 2.47$, $p = 0.036$). A significant difference was found between snakes from Ozaukee and Racine in the number of strikes ($p = 0.043$) when the litter variable was removed.

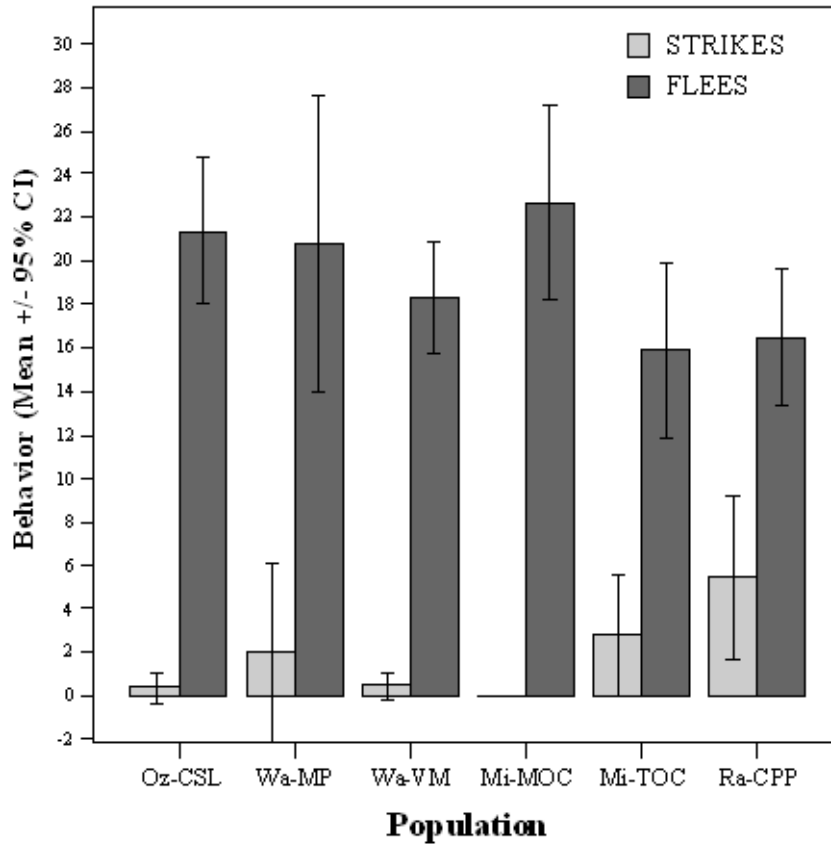


Figure 10. Behavioral differences in antipredator behavior of the populations of *Thamnophis butleri* in southeastern Wisconsin for strikes and flees two days post-partum. No significant population effects were found for strikes or flees when litter was nested within population (but see text).

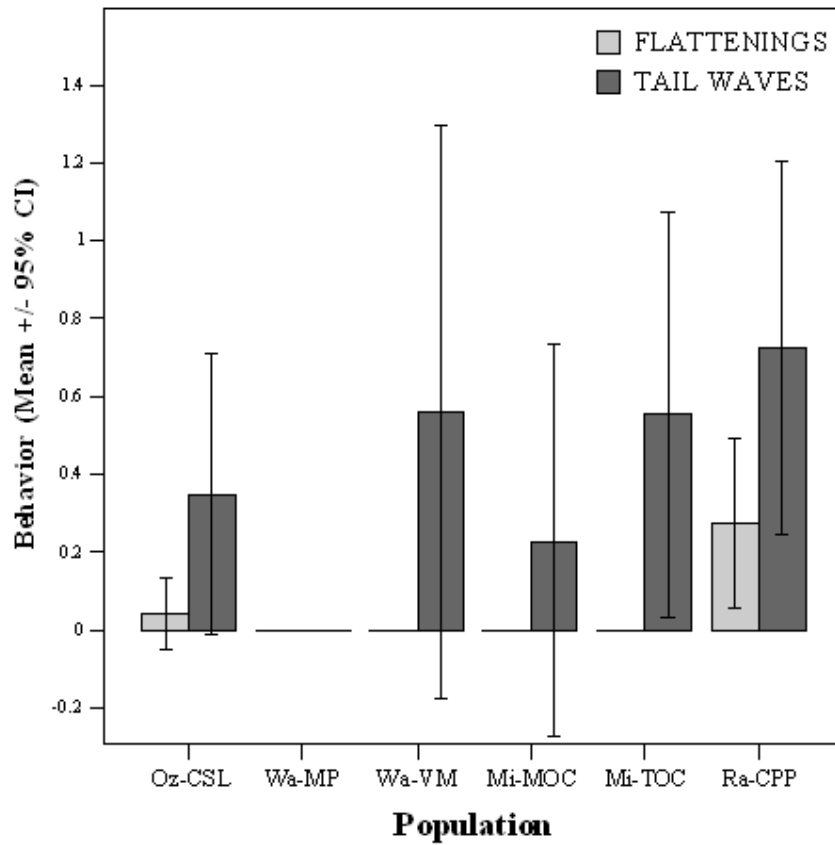


Figure 11. Behavioral differences in antipredator behavior of the populations of *Thamnophis butleri* in southeastern Wisconsin for flattenings and tail waves two days post-partum. No significant population effects were found for flattenings or tail waves.

Initial Test- Chi-Square Test Results

More *T. butleri* tail waved than *T. radix* ($\chi^2= 4.670$, $df= 1$, $n= 173$, $p= 0.031$) but no significance difference was found in the number of snakes that flattened ($\chi^2= 0.084$, $df= 1$, $n= 173$, $p= 0.771$ - see Table 7 for presence or absence of behaviors). Likewise, more MI/OH *T. butleri* tail waved than *T. radix* ($\chi^2= 12.489$, $df= 1$, $n= 46$, $p= 0.000$), but no significance difference was found in the number of snakes that flattened ($\chi^2= 0.087$, $df= 1$, $n= 46$, $p= 0.768$). There was no significant difference between the number of Wisconsin *T. butleri* and *T. radix* that tail waved ($\chi^2= 3.022$, $df= 1$, $n= 139$, $p= 0.082$) or flattened ($\chi^2= 0.075$, $df= 1$, $n= 139$, $p= 0.784$).

More MI/OH *T. butleri* tail waved than Wisconsin *T. butleri* ($\chi^2= 19.329$, $df= 1$, $n= 161$, $p= 0.000$), but no significance difference was found for flattenings ($\chi^2= 0.008$, $df= 1$, $n= 161$, $p= 0.929$).

Second Antipredator Test

In the second antipredator test, *T. radix* were more likely to strike ($F_{1,170}= 4.72$, $p= 0.031$, Figure 12) and flatten than *T. butleri* ($F_{1,170}= 4.87$, $p= 0.029$, Figure 13), and *T. butleri* continued to be more likely to flee than *T. radix* ($F_{1,170}= 6.64$, $p= 0.011$, see Figures 14). No significant differences were found in tail waves ($F_{1,170}= 1.61$, $p= 0.206$). *T. radix* subjects were also significantly more likely to strike than MI/OH *T. butleri* ($F_{1,46}= 16.68$, $p= 0.000$), as well as to flatten ($F_{1,46}= 12.36$, $p= 0.001$), while MI/OH *T. butleri* were more likely to flee ($F_{1,46}= 44.42$, $p= 0.000$). No significant differences were found for tail waving ($F_{1,46}= 1.33$, $p= 0.255$).

Table 7. Data from all *Thamnophis butleri* and *T. radix* study subjects of presence or absence of tail waves (tail) and flattenings (flat) two days (1st) and 22-25 days (2nd) post-partum antipredator tests. Chi-square tests were conducted with these data (see text for significance results).

Test: behavior	Presence of behavior	<i>T. radix</i>	All <i>T. butleri</i>	MI/OH <i>T. butleri</i>	Wisconsin <i>T. butleri</i>
1 st : Tail	no	12	115	14	101
	yes	0	46	20	26
1 st : Flat	no	11	151	32	119
	yes	1	10	2	8
2 nd : Tail	no	12	134	30	104
	yes	0	25	4	21
2 nd : Flat	no	9	151	34	117
	yes	3	8	0	8

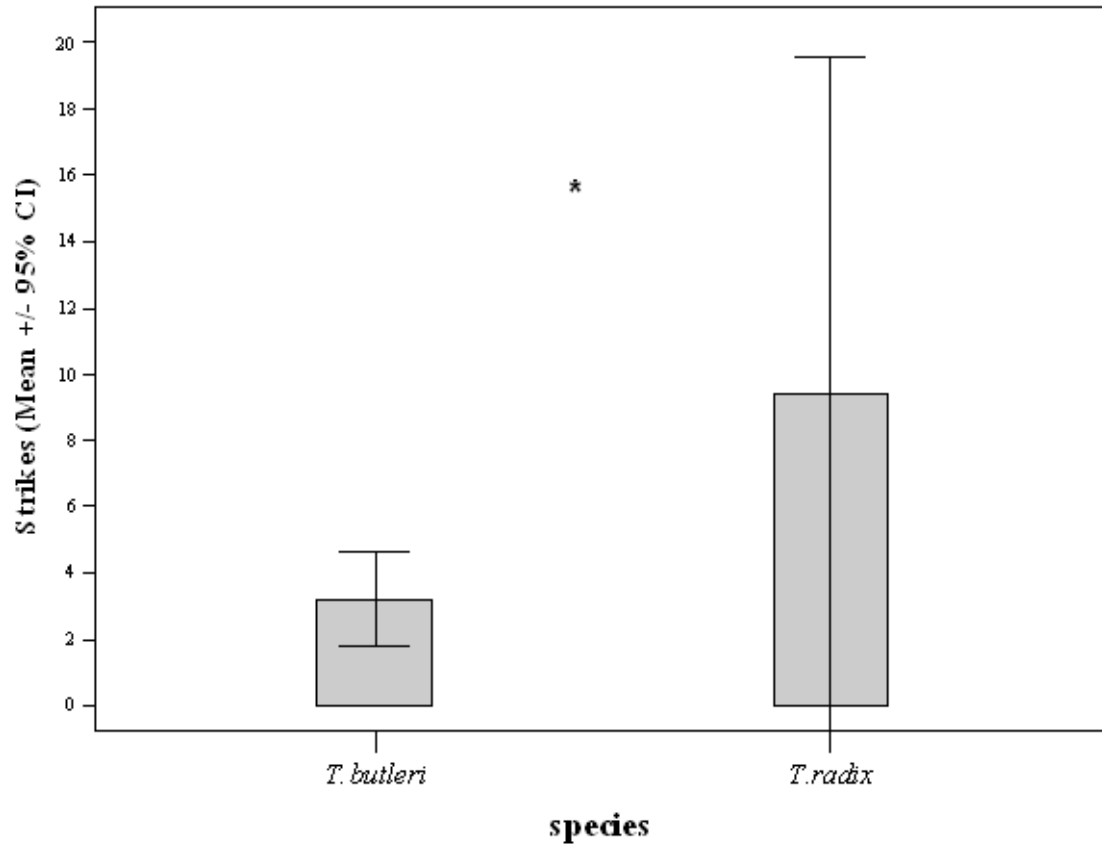


Figure 12. Comparisons of strikes of *Thamnophis butleri* and *T. radix* 22-25 days post-partum. Significant species effects were found.
* = $p \leq 0.05$

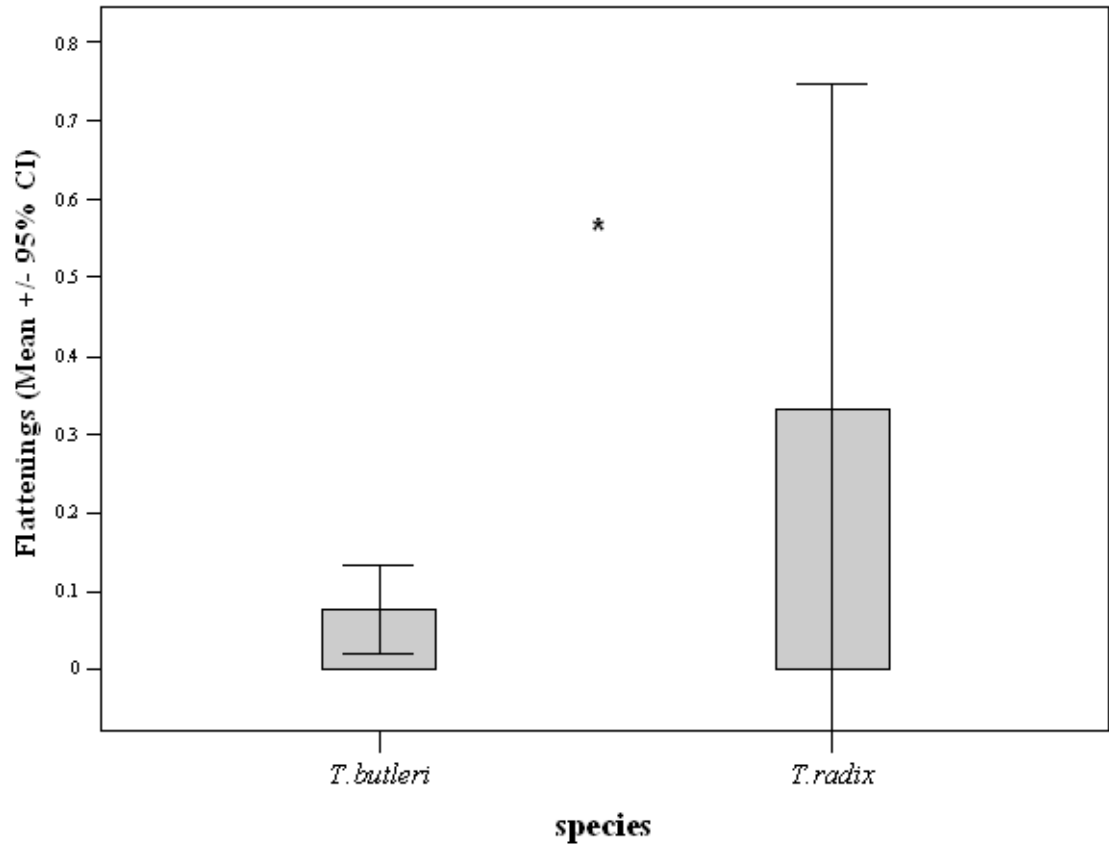


Figure 13. Comparisons of flattenings of *Thamnophis butleri* and *T. radix* 22-25 days post-partum. Significant species effects were found.
* = $p \leq 0.05$

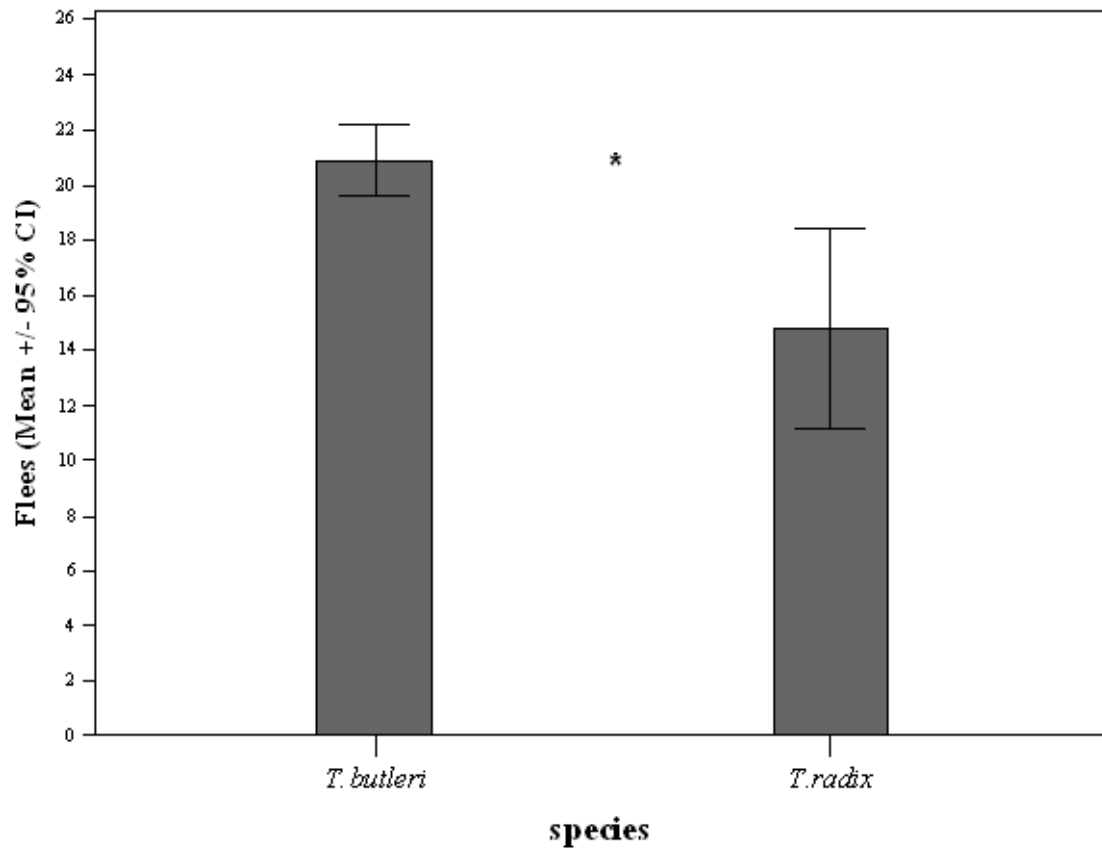


Figure 14. Comparisons of flees of *Thamnophis butleri* and *T. radix* 22-25 days post-partum. Significant species effects were found.
 * = $p \leq 0.05$

Thamnophis radix subjects were more likely to strike ($F_{1, 137} = 4.22$, $p = 0.042$) and flatten ($F_{1, 137} = 4.35$, $p = 0.039$) than Wisconsin *T. butleri* subjects. No significant differences were found in flees ($F_{1, 137} = 2.90$, $p = 0.091$) or tail waving ($F_{1, 137} = 1.75$, $p = 0.188$).

MI/OH *T. butleri* were more likely to flee ($F_{1, 159} = 55.37$, $p = 0.000$) and less likely to strike ($F_{1, 159} = 4.95$, $p = 0.028$) than were *T. butleri* from Wisconsin. No significant differences were found in tail waving ($F_{1, 159} = 0.003$, $p = 0.955$) or flattening ($F_{1, 159} = 1.81$, $p = 0.180$). Thus, overall, Wisconsin *T. butleri* were more similar to *T. radix* than were MI/OH *T. butleri* in the second antipredator test.

Populations of *T. butleri* from southeastern Wisconsin did not significantly differ in the number of strikes ($F_{5, 7} = 3.16$, $p = 0.086$), flees ($F_{5, 7} = 1.00$, $p = 0.490$), head/body flattening ($F_{5, 7} = 0.96$, $p = 0.500$), or tail waves ($F_{5, 7} = 6.70$, $p = 0.660$, see Figures 15, 16). I ran a separate ANOVA examining only Ozaukee and Racine population differences, and a significant difference was found in the number of strikes ($F_{1, 6} = 7.34$, $p = 0.037$).

As in the first antipredator test, a significant difference among Wisconsin populations was found in the number of strikes ($F_{5, 124} = 9.51$, $p = 0.000$) and flees ($F_{5, 124} = 3.43$, $p = 0.006$) when the nested litter variable was removed. The amount of flattening was also significantly different among the populations ($F_{5, 124} = 3.30$, $p = 0.008$) when litter was removed.

Second Antipredator Test- Chi-Square Test Results

A greater number of *T. radix* flattened when compared to *T. butleri* ($\chi^2 = 7.392$, $df = 1$, $n = 171$, $p = 0.007$, Table 7). No significance difference was found for tail waves ($\chi^2 = 2.210$, $df = 1$, $n = 171$, $p = 0.137$).

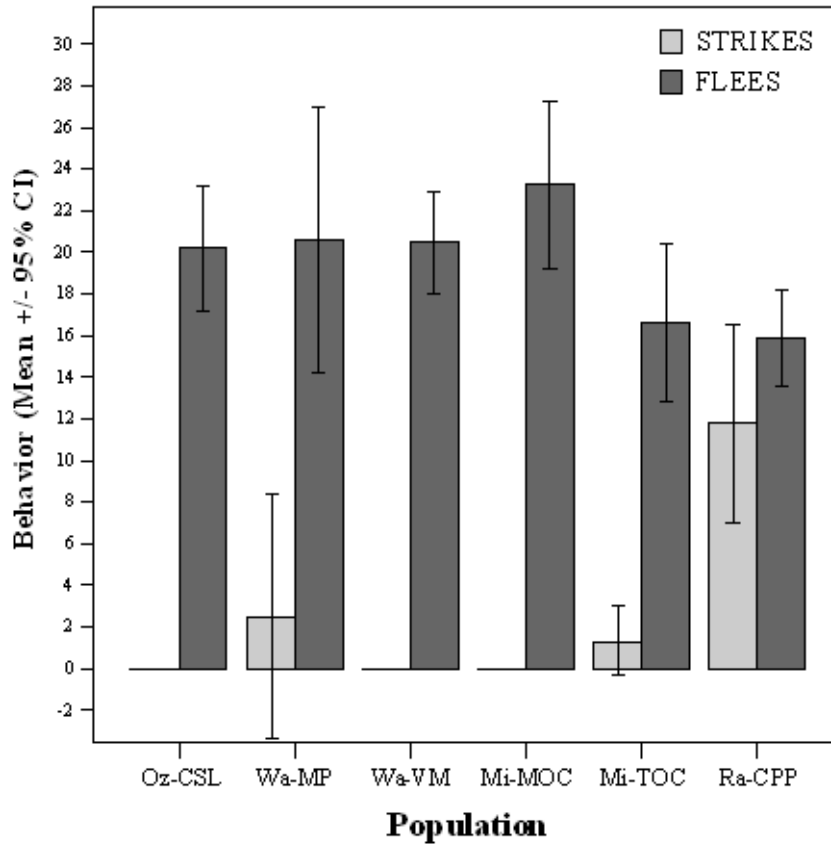


Figure 15. Behavioral differences of the populations of *Thamnophis butleri* in southeastern Wisconsin for strikes and flees 22-25 days post-partum. No significant population effects were found (see text for Ozaukee/Racine comparison).

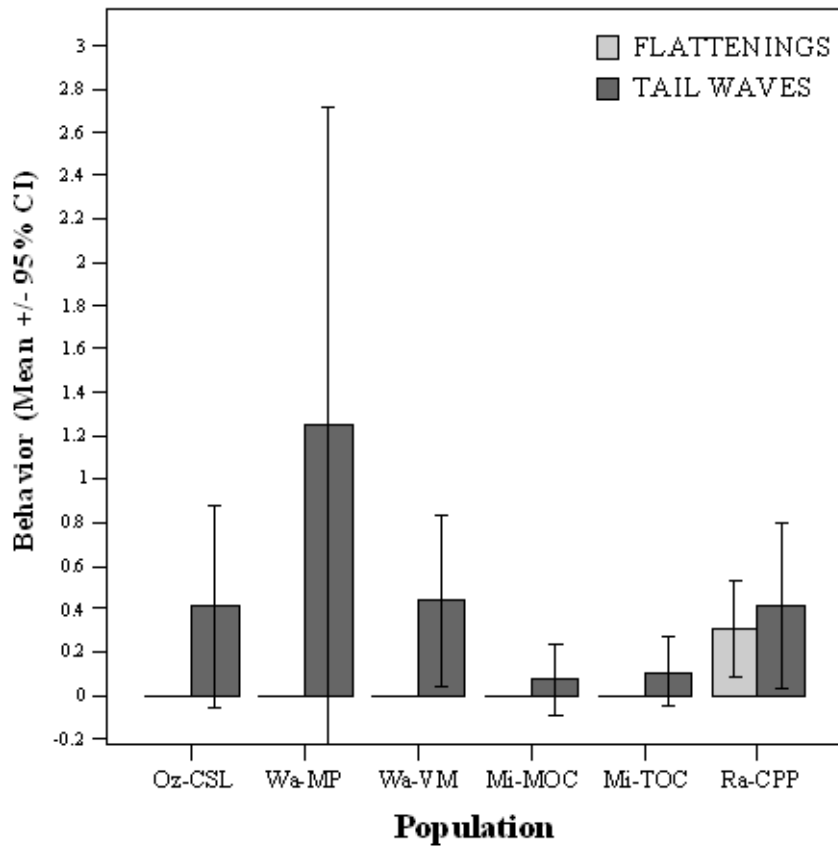


Figure 16. Behavioral differences of the populations of *Thamnophis butleri* in southeastern Wisconsin for flattenings and tail waves 22-25 days post-partum. No significant population effects were found.

Likewise, more *T. radix* flattened than MI/OH *T. butleri* ($\chi^2= 9.093$, $df= 1$, $n= 46$, $p= 0.003$), but no significance difference was found for tail waving ($\chi^2= 1.546$, $df= 1$, $n= 46$, $p= 0.214$).

More *T. radix* subjects flattened than did Wisconsin *T. butleri* ($\chi^2= 5.129$, $df= 1$, $n= 137$, $p= 0.024$), but no significance difference was found for tail waving ($\chi^2= 2.381$, $df= 1$, $n= 137$, $p= 0.123$).

No significant differences were found between MI/OH and Wisconsin *T. butleri* in the number of snakes that tail waved ($\chi^2= 0.511$, $df= 1$, $n= 159$, $p= 0.475$) or flattened ($\chi^2= 2.291$, $df= 1$, $n= 159$, $p= 0.130$).

Differences Between First and Second Antipredator Tests

A paired t-test, using only *T. butleri*, revealed significant differences between the first and second antipredator tests for strikes ($t= -2.001$, $df= 158$, $p= 0.047$) and tail waves ($t= 2.590$, $df= 158$, $p= 0.010$). The number of strikes was significantly higher in the second test and the number of tail waves was significantly lower in the second test than in the first test (see Figures 17, 18). None of the other behaviors showed a significant overall change between the two tests (flees: $t= 0.183$, $df= 158$, $p= 0.855$; flattening: $t= 0.391$, $df= 158$, $p= 0.696$).

Threat Level Differences

In the first antipredator test, *T. butleri* subjects showed a significant difference between the threat levels for fleeing ($F_{2, 152}= 242.94$, $p= 0.000$), but not for striking ($F_{2, 152}= 1.60$, $p= 0.206$, see Figure 19). Snakes were most likely to flee during the touching phase when compared to the other threat levels (touching-still: $p= 0.000$; touching-moving: $p= 0.000$), and snakes were more likely to flee during the still phase than the

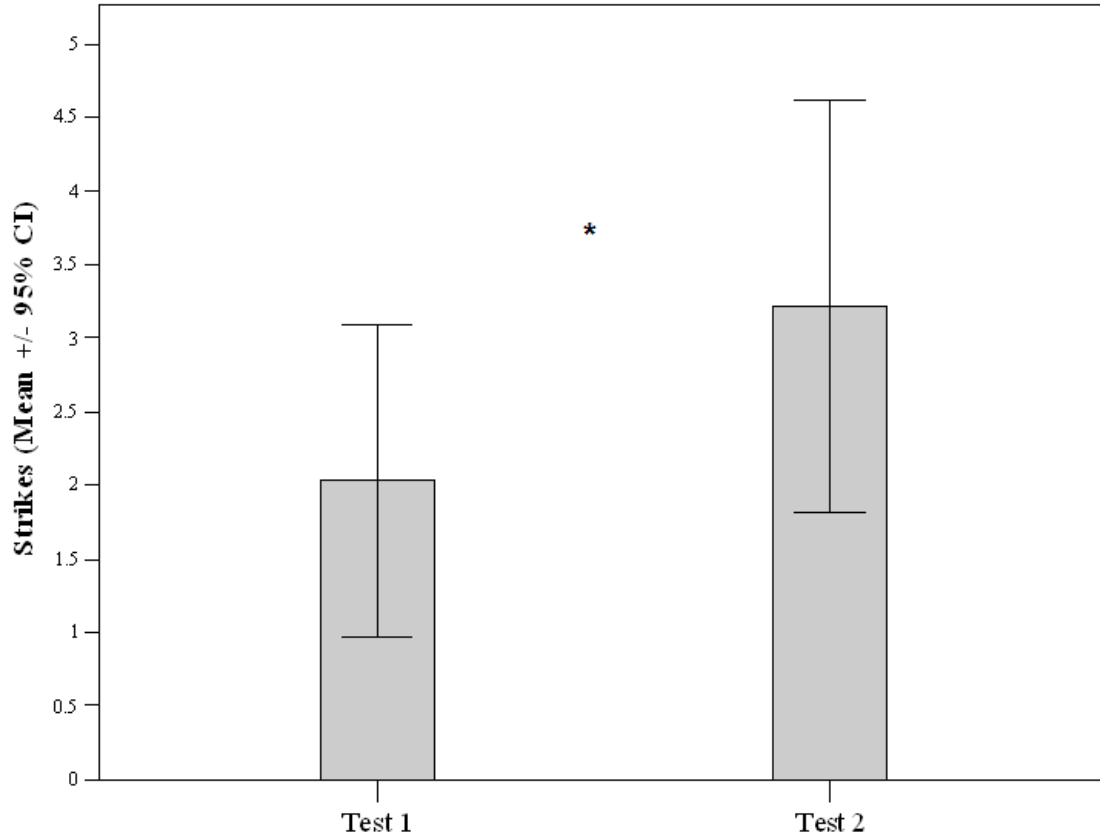


Figure 17. Strike comparisons of *Thamnophis butleri* in the first and second antipredator tests. A significant difference was found between the two tests.

* = $p \leq 0.05$

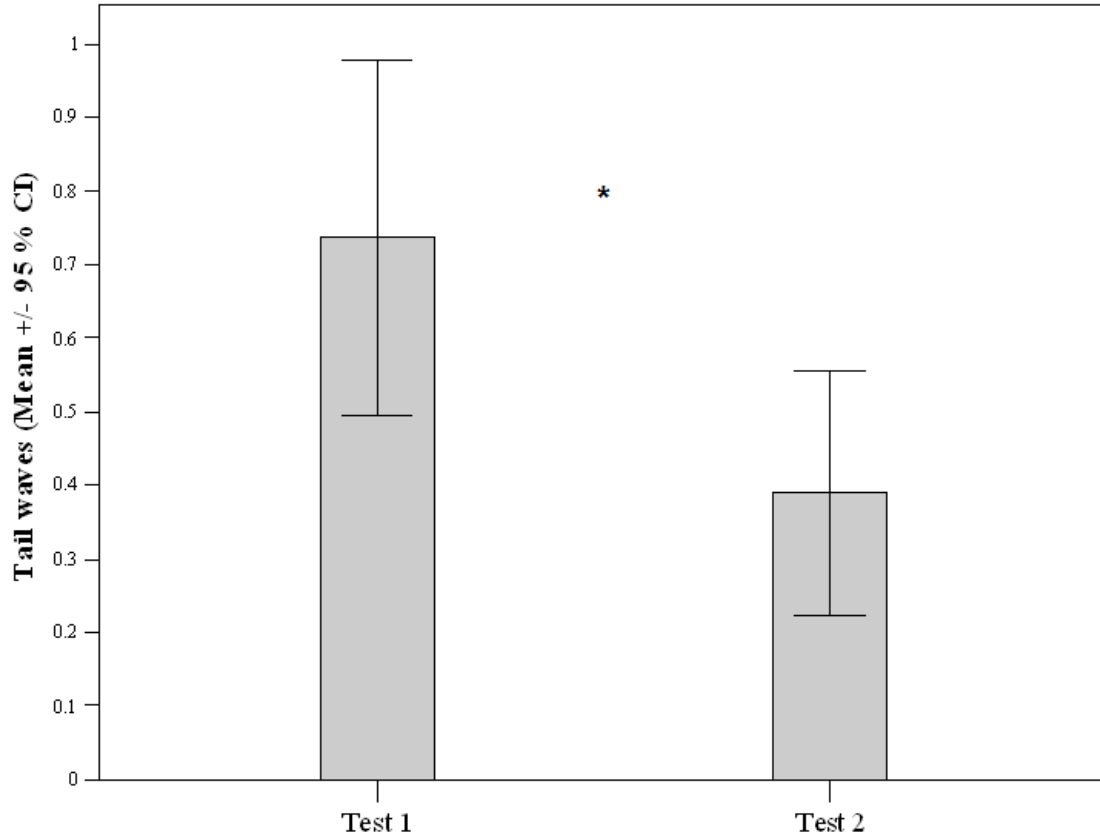


Figure 18. Tail wave comparisons of *Thamnophis butleri* in the first and second antipredator tests. A significant difference was found between the two tests.

* = $p \leq 0.05$

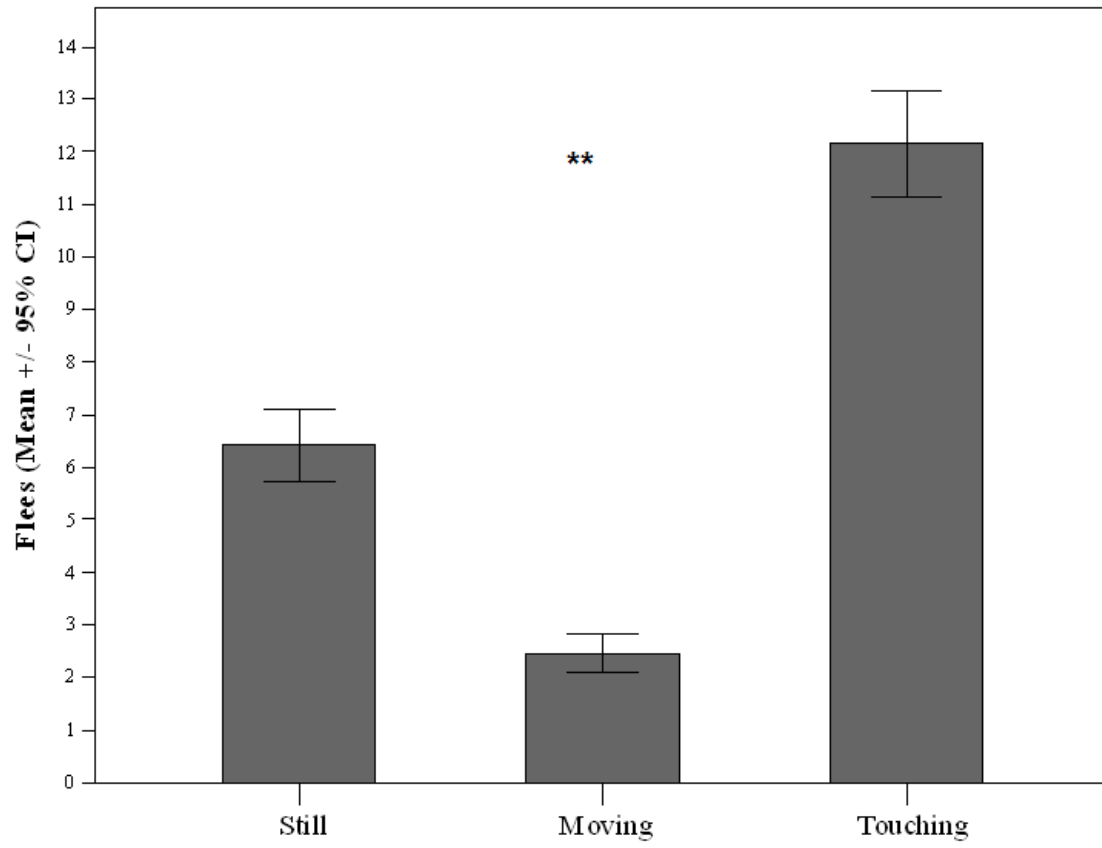


Figure 19. Comparisons of threat levels by *Thamnophis butleri* for fleeing two days post-partum. A significant difference was found between the threat levels.
** = $p \leq 0.01$

moving phase ($p= 0.000$). *Thamnophis radix* subjects showed similar results in that a significant difference was found between the threat levels for fleeing ($F_{2, 10}= 33.75$, $p= 0.000$), but not for striking ($F_{2, 10}= 0.33$, $p= 0.727$). *Thamnophis radix* subjects were more likely to flee during the touching phase than the moving phase ($p= 0.000$), but there was no significant difference between flees in the touching phase versus the still phase. Snakes were also more likely to flee during the still phase than the moving phase ($p= 0.000$).

In the second antipredator test, *T. butleri* subjects showed significant differences between the threat levels for fleeing ($F_{2, 150}= 387.69$, $p= 0.000$, see Figure 20), but not striking ($F_{2, 150}= 1.60$, $p= 0.205$). Snakes were most likely to flee during the touching phase compared to the other threat levels (touching-still: $p= 0.000$; touching-moving: $p= 0.000$) and snakes were more likely to flee during the still phase than the moving phase ($p= 0.000$). *Thamnophis radix* subjects showed significant differences between the threat levels for fleeing ($F_{2, 10}= 39.93$, $p= 0.000$), but not striking ($F_{2, 10}= 2.25$, $p= 0.156$). *Thamnophis radix* subjects were more likely to flee during the touching phase than the moving phase ($p= 0.000$), but there was no significant difference between flees in the touching phase versus the still phase. Snakes were also more likely to flee during the still phase than the moving phase ($p= 0.000$).

Chemosensory Tests

Very few differences were found between the first and second presentations of the three stimuli during the same test session; therefore, I will present the results of the average between the two presentations. Also, although a main effect of order (regardless of stimulus) was found ($F_{2, 1023}= 3.958$, $p= 0.019$), order of stimulus presentation was

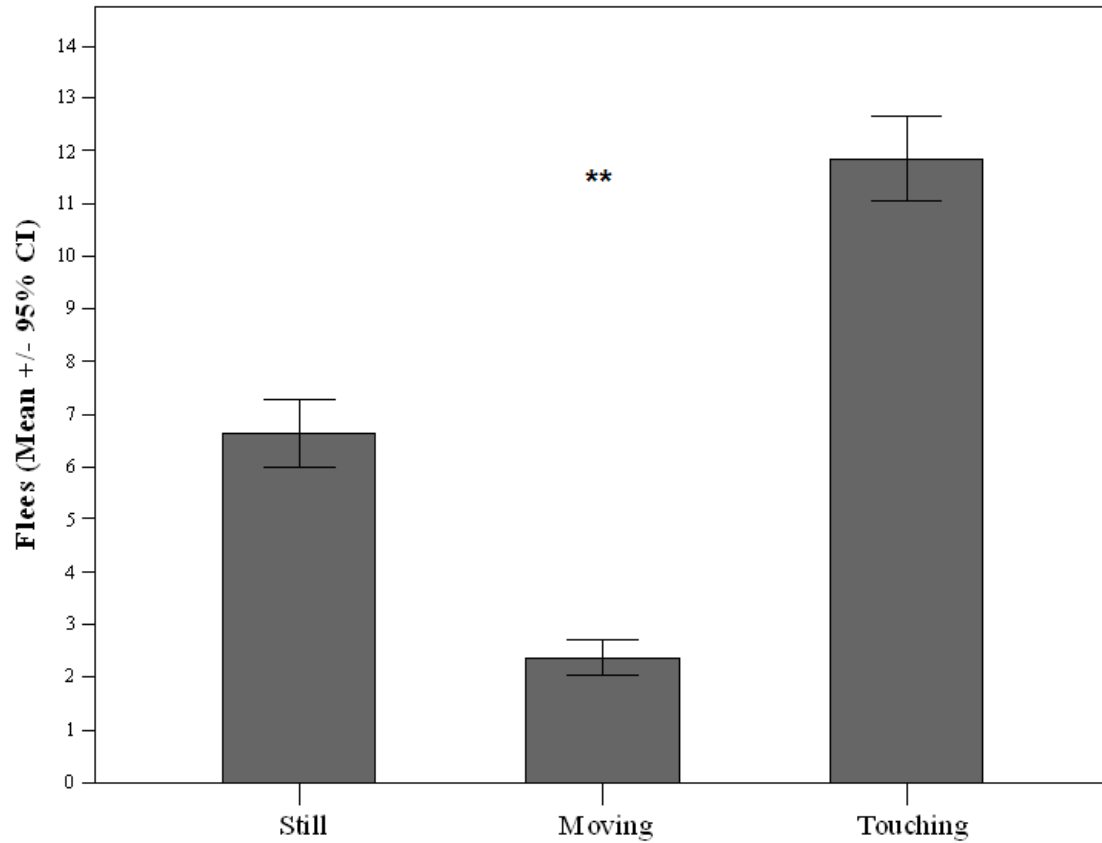


Figure 20. Comparisons of threat levels by *Thamnophis butleri* for fleeing 22-25 days post-partum. A significant difference was found between the threat levels.

** = $p \leq 0.01$

balanced throughout and therefore should not be a major source of error.

Order effects were also found in the responses to the stimuli ($F_{4, 1023} = 2.92$, $p = 0.020$). Responses to water differed according to the order of the presentation of the stimuli. If the water stimulus was presented first, the TFAS was significantly higher than if it was presented second ($p = 0.036$) or third ($p = 0.003$), which possibly reflects habituation (Burghardt 1969). The responses to fish also differed according to the order of the presentation of stimuli, with significantly higher TFAS if the fish stimulus was presented second, versus when it was presented first ($p = 0.024$) or third ($p = 0.002$).

Overall Response Differences

An overall response difference to the stimuli by *T. butleri* subjects was found ($F_{2, 151} = 209.39$, $p = 0.000$), with worm and fish stimuli being responded to much more than the water stimulus, but with no significant difference in the TFAS scores for worm and fish (see Figure 21).

Attack Differences

A chi-squared test using all *T. butleri* subjects revealed a significant difference in the number of attacks to the stimuli ($\chi^2 = 18.47$, $df = 2$, $n = 161$, $p = 0.000$), with more attacks to fish than to worm. Because of the low instance of attack by populations, analyses for population differences were run by county/area and not split into population. There was a significant difference in the number of attacks elicited by each county/area ($\chi^2 = 23.93$, $df = 6$, $n = 173$, $p = 0.001$). Snakes from Racine county produced the greatest number of attacks, with 65% of the attacks given to fish, while Ozaukee county snakes attacked the worm stimulus more often (see Table 8). Snakes from Waukesha county

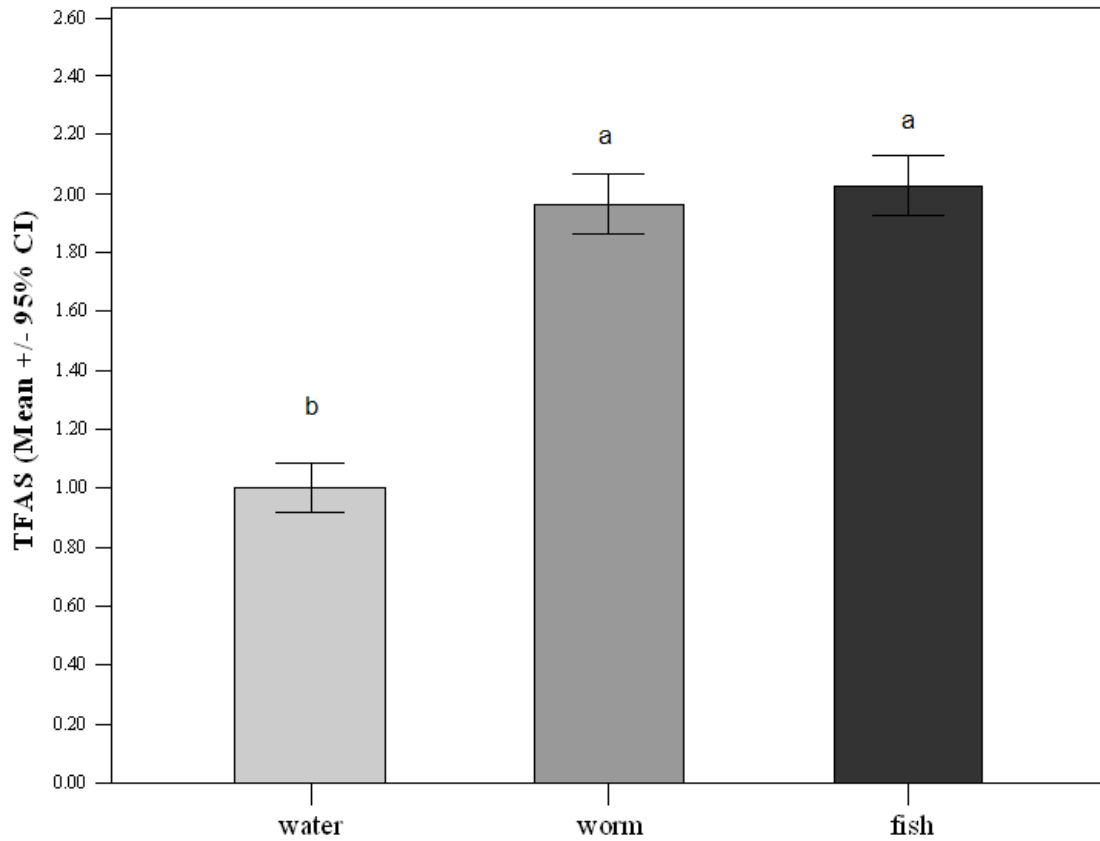


Figure 21. Comparisons of the responses of *Thamnophis butleri* to the different stimuli in the average of the two chemosensory stimuli presentations. There was an overall significant difference in TFAS to the different stimuli. Columns labeled with different letters are significantly different ($p \leq 0.01$).

Table 8. Number of attacks elicited by *Thamnophis butleri* and *T. radix* to the prey stimuli using all subjects.

Population	Total No. of attacks	Attacks to worm	Attacks to fish
MI	0	0	0
OH	0	0	0
Milwaukee (WI)	1	0	1
Ozaukee (WI)	6	4	2
Racine (WI)	17	6	11
Waukesha (WI)	7	2	5
Will (IL)	4	2	2
TOTAL:	35	14	21

attacked the fish stimulus more often and snakes from Will county attacked each stimulus equally. Snakes from Milwaukee county, Sterling, and Toledo rarely (if ever) attacked prey stimuli.

There was no significant difference in the number of attacks to worm between Ozaukee and Racine snakes ($\chi^2 = 0.063$, $df = 1$, $n = 63$, $p = 0.803$). However, there was a difference in the number of attacks to fish between Ozaukee and Racine snakes ($\chi^2 = 5.076$, $df = 1$, $n = 63$, $p = 0.024$), with Racine snakes attacking the fish more frequently.

TFAS Response Differences

There were no significant differences between *T. radix* and all *T. butleri* subjects in the responses to the stimuli for the average of the two presentations ($F_{1, 170} = 0.51$, $p = 0.477$). However, there was a significant difference when *T. radix* and MI/OH *T. butleri* responses were compared ($F_{1, 44} = 4.00$, $p = 0.052$). This was based only on a marginally significant difference in the responses of the two groups towards the water stimulus ($p = 0.058$), with *T. radix* showing a higher response towards water. The responses to the other stimuli were not significantly different.

There were no significant differences between *T. radix* and Wisconsin *T. butleri* in the responses to the different stimuli ($F_{1, 136} = 0.13$, $p = 0.721$), but there was a significant difference in Michigan/Ohio and Wisconsin *T. butleri* in the responses to the different stimuli ($F_{1, 158} = 6.62$, $df = 1$, $p = 0.011$). Wisconsin *T. butleri* responded significantly more to both water and fish than MI/OH *T. butleri* (water: $p = 0.038$; fish: $p = 0.008$).

No significant differences were found among the populations of southeastern Wisconsin in responses to water, worms or fish (water: $F_{5, 7} = 0.371$, $p = 0.853$;

worm: $F_{5, 7} = 0.81$, $p = 0.577$; fish: $F_{5, 7} = 1.80$, $p = 0.235$, Figure 22) when litter was nested within population. However, when population differences were examined without nesting litter within population, a significant difference was found among the populations for response to worm ($F_{5, 120} = 5.85$, $p = 0.000$) and fish ($F_{5, 120} = 5.18$, $p = 0.000$).

Relative Chemosensory Preferences

A multivariate analysis of variance revealed a significant effect among the populations of southeastern Wisconsin for the average W-F TFAS ($F = 3.93$, $df = 5$, $p = 0.002$, see Figure 23). Snakes from Ozaukee had a positive W-F TFAS score, meaning that they preferred worms, while snakes from Racine showed a negative W-F TFAS, meaning that these snakes preferred fish. A Tukey post-hoc test revealed a significant difference between the two populations ($p = 0.002$).

Prey Preference Test

Overall, in the live prey choice test, 73% of the snakes chose worms first, 20% chose fish first, and 7% never chose a prey item (Table 9). Half of the snakes from Sterling chose fish first, while all of the snakes from Toledo chose worm first. Of Wisconsin *T. butleri*, 68% of snakes from Racine county chose worm first, 77% of snakes from Milwaukee county chose worm first, 82% of snakes from Ozaukee county chose worm first, and 97% of snakes from Waukesha county chose worms first. In fish choice, 28% of Racine county snakes chose fish first, followed by snakes from Ozaukee county (14%), Milwaukee county (13%), and Waukesha county (3%).

Correlations among Behavioral and Morphological Measures

Significant correlations were found between mass and worm TFAS ($r = -0.408$, $N = 161$, $p = 0.000$) and mass and fish TFAS ($r = -0.297$, $N = 161$, $p = 0.000$).

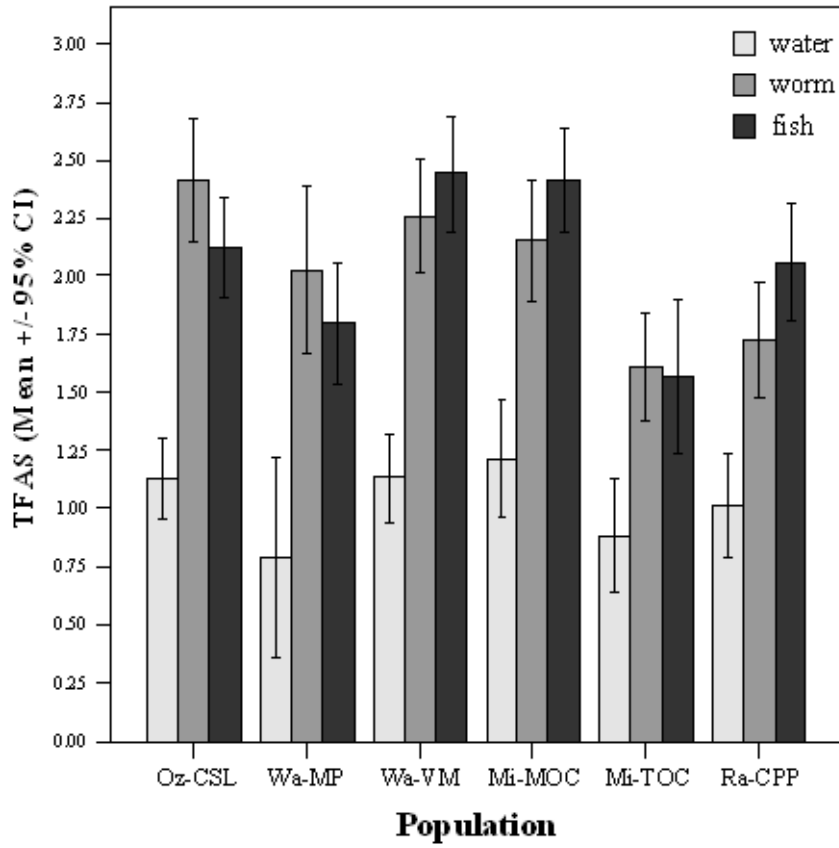


Figure 22. Comparison of tongue-flick attack scores (TFAS) to the three stimuli in populations of *Thamnophis butleri* from southeastern Wisconsin in the average of the two presentations.

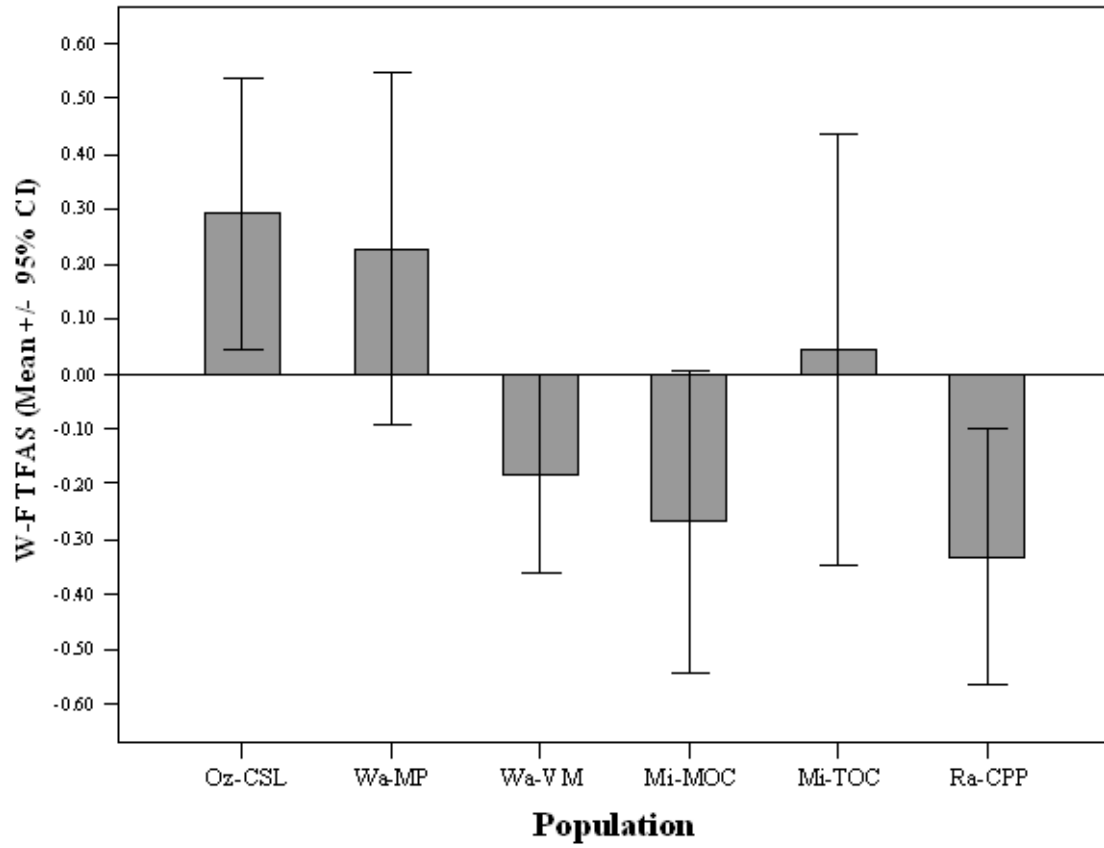


Figure 23. Comparison of the worm-fish tongue-flick attack scores (W-F TFAS) in the populations of *Thamnophis butleri* from southeastern Wisconsin in the average of the two presentations. A W-F TFAS value above the line represents a bias towards worm, and below the line, to fish. There was a significant population effect for these biases.

Table 9. First prey choice of *Thamnophis butleri* and *T. radix* by county using all subjects. (Data are for first prey choice, so prey items were not necessarily chosen on first prey presentation)

County	n	Worm	Fish	Neither
Sterling (MI)-2 litters	18	3	9	6
Toledo (OH)-1 litter	16	16	0	0
Milwaukee (WI)-3 litters	31	24	4	3
Ozaukee (WI)-3 litters	22	18	3	1
Racine (WI)-4 litters	40	27	11	2
Waukesha (WI)-3 litters	33	32	1	0
Will (IL)-2 litters	12	6	6	0

Although several of the other behaviors showed significant correlations, the correlations were not significant after correcting for multiple comparisons using the Holm's method.

Table 10 shows the correlation matrix of examined behaviors.

Discriminant Function Analysis

I used only behavioral data that seemed to discriminate well among the populations and species. These included antipredator behaviors (strikes and flees) and chemosensory responses (TFAS to worm and fish, and W-F TFAS). [The W-F TFAS variable was dropped from the analysis because it failed to meet the tolerance test (it is a function of the worm and fish TFAS)].

I first conducted a discriminant function analysis examining all six populations of Wisconsin *T. butleri* in order to determine how many could be correctly placed using only behavioral data. In this test, there was a significant effect of the discriminant function analysis using the four chosen behavioral variables ($\lambda = 0.575$, $\chi^2 = 66.369$, $df = 20$, $p = 0.000$). The contributions of each variable to the analysis are listed in Table 11. This analysis correctly classified 38.1% of the cases, as compared to less than 17% classification by chance (Table 12).

I then conducted two separate discriminant function analyses, one with snakes from Ozaukee and Racine (2 populations) and the other with snakes from Milwaukee and Waukesha counties (4 populations). Table 13 lists the contributions of each variable to the analysis for the Ozaukee/Racine test. There was a significant effect of the discriminant function analysis for the Ozaukee/Racine test ($\lambda = 0.687$, $\chi^2 = 21.794$, $df = 4$, $p = 0.000$), with 75.8% of the cases correctly classified (Table 14).

Table 10. Correlation matrix of behaviors exhibited by *Thamnophis butleri*. Numbers represent Pearson's Correlation coefficient.

	SVL	Body cond. index	Strikes	Flees	Water-TFAS	Worm-TFAS	Fish-TFAS	Total attacks to chem. stimuli
Mass	0.805**	0.543**	0.037	0.155*	-0.215**	-0.408**	-0.297**	-0.184*
SVL		-0.048	0.043	0.161*	-0.157*	-0.306**	-0.247**	-0.100
Body cond. index			0.152	0.012	-0.124	-0.255**	-0.158*	-0.180*
Strikes				-0.098	-0.038	-0.053	0.057	0.153*
Flees					0.045	0.049	-0.065	-0.029
Water TFAS						0.457**	0.370**	0.196*
Worm- TFAS							0.565**	0.399**
Fish- TFAS								0.472**

* p < 0.05

** p < 0.01

Numbers in bold face are significant after Holm's correction method for multiple comparisons.

Table 11. Standardized canonical discriminant function coefficients for *Thamnophis butleri*. The coefficients convey the contributions of each variable to the analysis.

Behavior variable	Partial contribution
Strikes	-0.542
Flees	0.394
Average worm TFAS	0.677
Average fish TFAS	0.178

Table 12. Classification results for the discriminant function analysis for *Thamnophis butleri* from southeastern Wisconsin. The left population column is the original population and the top population row is the population that the discriminant function analysis predicted using the behavioral variables. This analysis correctly classified 38.1% of the cases.

		Predicted Group Membership							
		Mil-MOC	Mil-TOC	Oz-CSL	Ra-CPP	Wa-MP	Wa-VM	Total	
Original	Count	Mil-MOC	1	0	4	2	0	6	13
		Mil-TOC	0	3	3	12	0	0	18
		Oz-CSL	1	0	14	7	0	1	23
		Ra-CPP	1	7	6	17	0	8	39
		Wa-MP	0	1	4	3	0	0	8
		Wa-VM	0	0	4	8	0	13	25

Table 13. Standardized canonical discriminant function coefficients for *Thamnophis butleri* from Racine and Ozaukee. The coefficients convey the contributions of each variable to the analysis.

Behavior variable	Partial contribution
Strikes	-0.410
Flees	0.465
Average worm TFAS	0.979
Average fish TFAS	-0.509

Table 14. Classification results for the discriminant function analysis for *Thamnophis butleri* from Ozaukee and Racine. The left population column is the original population and the top population row is the population that the discriminant function analysis predicted using the behavioral variables. This analysis classified 75.8% of the cases correctly.

		Population	Predicted Group		Membership	Total
			Oz- CSL	Ra- CPP		
Original	Count	Oz- CSL	15	8		23
		Ra- CPP	7	32		39

Table 15 lists the contributions of each variable to the analysis for the Milwaukee/Waukesha test. There was a significant effect of the discriminant function analysis for this test ($\lambda = 0.462$, $\chi^2 = 45.527$, $df = 12$, $p = 0.000$), which classified 62.5% of the cases correctly (Table 16). Thus, behavioral data were effective in separating snakes from populations only separated by 5-58 km.

Table 15. Standardized canonical discriminant function coefficients for *Thamnophis butleri* from Milwaukee and Waukesha. The coefficients convey the contributions of each variable to the analysis.

Behavior variable	Partial contribution
Strikes	-0.540
Flees	0.392
Average worm TFAS	0.214
Average fish TFAS	0.810

Table 16. Classification results for the discriminant function analysis for *Thamnophis butleri* from Milwaukee and Waukesha. The left population column is the original population and the top population row is the population that the discriminant function analysis predicted using the behavioral variables. This analysis classified 62.5% of the cases correctly.

		Predicted Group Membership				Total	
		Mil-MOC	Mil-TOC	Wa-MP	Wa-VM		
Original	Count	Population					
		Mil-MOC	5	0	0	8	13
		Mil-TOC	1	15	1	1	18
		Wa-MP	2	2	0	4	8
		Wa-VM	2	2	1	20	25

4. DISCUSSION

The taxonomic status of *T. butleri* and *T. radix* is still unresolved across southeastern Wisconsin. This study addresses this taxonomic issue with the use of behavior. Several hypotheses have been put forth as to the interactions that occur when two species are sympatric. Character displacement can lead to the origin of novel phenotypes in response to competition among populations (Marko 2005). However, recent genetic and morphological evidence do not support this hypothesis regarding *T. butleri* and *T. radix* in southeastern Wisconsin.

Another hypothesis emphasizes the importance of local ecology. In this hypothesis, the local ecology drives the behaviors of the species. However, although this hypothesis cannot be ruled out, it does not explain the clinal variation over such a short distance.

The focus of this study was on the hypothesis of hybridization between *T. butleri* and *T. radix* in southeastern Wisconsin. Morphological and genetic evidence support the hypothesis of gene flow between *T. butleri* and *T. radix*. If my data support the hypothesis of hybridization in these species, I would predict that: (1) the behaviors of *T. butleri* in Wisconsin will differ from the behaviors of Michigan and Ohio *T. butleri* in the direction of being more like *T. radix*; (2) the behaviors of *T. butleri* from the different counties and populations in Wisconsin will differ from one another; (3) *Thamnophis butleri* from the northern part of the Wisconsin range, further removed from the range of *T. radix*, will show behaviors more like Michigan *T. butleri* and less like *T. radix* than do *T. butleri* from the southern part of their range in Wisconsin.

Several differences were found among the species and populations examined in this study, including differences in morphology, antipredator behavior, and feeding behavior. I will discuss results from each section, and then present a general discussion at the end of this section. The small sample size of *T. radix* subjects from only one population, compared to *T. butleri* subjects, could have had an effect on the species comparisons. Therefore, these are in particular need of replication. Tables 17-20 summarize the main findings related to the hypotheses tested.

Morphology

Thamnophis radix were longer and heavier than *T. butleri* when SVL was not used as a covariate. This is consistent with past research documenting species differences in neonate size (King et al. 1999).

Prediction 1

MI/OH *T. butleri* were found to be significantly longer and heavier than Wisconsin *T. butleri*, which does not support my first prediction because *T. radix* was the longer and heavier species.

A body condition index for each individual was calculated using an equation developed by Rivas (2000). The higher condition index found in *T. butleri* snakes over *T. radix* snakes could simply indicate that *T. butleri* is the more stocky species. However, MI/OH *T. butleri* had a significantly higher body condition index than did Wisconsin *T. butleri*, suggesting that Wisconsin *T. butleri* are closer to *T. radix* in body shape.

Predictions 2, 3

Population differences within the snakes from southeastern Wisconsin were found for SVL, mass, and tail length. Snakes from Racine had significantly longer SVLs than

Table 17. Intraspecific and interspecific morphological comparisons of *Thamnophis butleri* and *T. radix*. The species/population listed is the species/population with the highest value. An asterisk lists statistical significance. Differences that are close to significance or in the hypothesized direction are included in parentheses.

	<i>T. radix</i> vs. <i>T. butleri</i>	<i>T. radix</i> vs. MI/OH <i>T. butleri</i>	<i>T. radix</i> vs. Wisconsin <i>T. butleri</i>	MI/OH vs. Wisconsin <i>T. butleri</i>	Wisconsin <i>T. butleri</i> populations	Ozaukee vs. Racine
SVL	<i>T. radix</i> *	<i>T. radix</i> *	<i>T. radix</i> *	MI/OH*	*	Racine*
Mass	<i>T. radix</i> *	<i>T. radix</i> *	<i>T. radix</i> *	MI/OH*	*	Racine*
Cond.	<i>T. butleri</i> *	MI/OH*	Wisconsin*	MI/OH*	*	ns

Table 18. Intraspecific and interspecific antipredator comparisons of *Thamnophis butleri* and *T. radix* for the first test. The species/population listed is the species/population with the highest value. An asterisk lists statistical significance. Differences that are close to significance or in the hypothesized direction are included in parentheses.

	<i>T. radix</i> vs. <i>T. butleri</i>	<i>T. radix</i> vs. MI/OH <i>T. butleri</i>	<i>T. radix</i> vs. Wisconsin <i>T. butleri</i>	MI/OH vs. Wisconsin <i>T. butleri</i>	Wisconsin <i>T. butleri</i> populations	Ozaukee vs. Racine
Strikes	<i>T. radix</i> *	<i>T. radix</i> *	<i>T. radix</i> *	ns	ns	ns (Rac)
Flees	<i>T. butleri</i> *	MI/OH*	Wisconsin*	MI/OH*	ns	ns (Oza)

Table 19. Intraspecific and interspecific antipredator comparisons of *Thamnophis butleri* and *T. radix* for the second test. The species/population listed is the species/population with the highest value. An asterisk lists statistical significance. Differences that are close to significance or in the hypothesized direction are included in parentheses.

	<i>T. radix</i> vs. <i>T. butleri</i>	<i>T. radix</i> vs. MI/OH <i>T. butleri</i>	<i>T. radix</i> vs. Wisconsin <i>T. butleri</i>	MI/OH vs. Wisconsin <i>T. butleri</i>	Wisconsin <i>T. butleri</i> populations	Ozaukee vs. Racine
Strikes	<i>T. radix</i> *	<i>T. radix</i> *	<i>T. radix</i> *	Wisconsin*	ns	Racine*
Flees	<i>T. butleri</i> *	MI/OH*	ns	MI/OH*	ns	ns (Oza)

Table 20. Intraspecific and interspecific chemosensory comparisons of *Thamnophis butleri* and *T. radix*. The species/population listed is the species/population with the highest value. An asterisk lists statistical significance. Differences that are close to significance or in the hypothesized direction are included in parentheses.

	<i>T. radix</i> vs. <i>T. butleri</i>	<i>T. radix</i> vs. MI/OH <i>T. butleri</i>	<i>T. radix</i> vs. Wisconsin <i>T. butleri</i>	MI/OH vs. Wisconsin <i>T. butleri</i>	Wisconsin <i>T. butleri</i> populations	Ozaukee vs. Racine
Overall TFAS	ns	*	ns	*	ns	ns
Water TFAS	--	ns (<i>T. radix</i>)	--	Wisconsin*	--	--
Worm TFAS	--	--	--	--	--	ns (Oza)
Fish TFAS	--	--	--	Wisconsin*	--	--
W-F TFAS	--	--	--	--	*	Oza*(W)

snakes from Ozaukee, but no consistent order was apparent between the other populations within the area. Snakes from Racine weighed significantly more than snakes from Ozaukee, which was the only significant difference found in mass. Snakes from Racine also had significantly longer tail lengths than snakes from Ozaukee and Waukesha-Vernon Marsh (see Appendix p. 94).

Snakes from both Milwaukee populations had higher body condition indices than snakes from all other populations in southeastern Wisconsin. This difference in condition could be due to a variety of factors, including genetic effects, feeding history, environment, and incubation temperature (before the pregnant females were brought into the laboratory). However, the Milwaukee animals are from the center of the range of the species in Wisconsin and thus could be less stressed because of superior habitat than populations at the northern, southern, or western limits of its range.

Other Findings

I found several significant correlations between *T. butleri* adult females and offspring. A significant correlation was found between pre-partum mass and the total number of offspring and between pre-partum mass and the number of live offspring. Maternal SVL also significantly correlated with the total number of offspring, as well as the number of live offspring. Ford and Killebrew (1983) also found that larger female (higher SVL) *T. butleri* produced larger clutch sizes. They also found that larger females produced heavier clutches (Ford and Killebrew 1983), which I did not find with my subjects. Albright (2001) found a significant positive correlation between dam mass and the number of offspring and a significant negative correlation between offspring mass

and the number of offspring in a litter. I did not find, however, a significant correlation between offspring mass and the number of offspring in a litter.

Antipredator Behavior

Prediction 1

In the first antipredator test, *T. radix* were more likely to strike than were MI/OH *T. butleri*, as well as Wisconsin *T. butleri*. There were no significant differences between MI/OH *T. butleri* and Wisconsin *T. butleri* in the number of strikes, but Wisconsin snakes struck more than MI/OH snakes. MI/OH *T. butleri* were significantly more likely to flee than were *T. radix* and Wisconsin *T. butleri*. These results indicate that Wisconsin *T. butleri* exhibit antipredator behaviors more like *T. radix* than like MI/OH *T. butleri*.

The data in the second antipredator test yielded similar results. *T. radix* snakes were more likely to strike than MI/OH *T. butleri*, and MI/OH *T. butleri* snakes were more likely to flee. This time, however, there was no significant difference in the amount of striking by *T. radix* and Wisconsin *T. butleri*. Also, Wisconsin *T. butleri* were significantly more likely to strike than were MI/OH snakes. These results strongly support my first prediction.

Predictions 2, 3

The most interesting data to examine were the differences in antipredator behavior between the counties in southeastern Wisconsin. Although I did not find significant differences in the behaviors when litter was nested within population, there were noticeable differences in the antipredator behaviors, and many of these were significant when the litter variable was removed from the analysis. Snakes from Racine, the closest to the range of *T. radix*, exhibited the most aggressive behaviors. Racine

snakes were more likely to strike than were snakes from the other populations in southeastern Wisconsin in the first antipredator test. Racine snakes also fled less often than most of the populations, leading to the conclusion that snakes from this area are more likely to exhibit aggressive antipredator behaviors.

In the first antipredator test, snakes from Ozaukee, which I hypothesized to behave more like *T. butleri* from other areas, performed a lower number of strikes than snakes from Racine, but this difference was not significant unless examined without the litter variable. Also, although the differences were not significant, snakes from Ozaukee performed more flees than the snakes from Racine. However, snakes from Milwaukee-Menard's Oak Creek performed the most flees, and snakes from Milwaukee-Target Oak Creek performed the lowest number of flees. Both of these populations are from southern Milwaukee county and close to the Racine population.

In the second antipredator test, again, no significant population differences were found among the populations of southeastern Wisconsin when litter was nested within population. When population differences were examined without the litter variable, highly significant differences were found for striking, fleeing, and flattening. Also, a significant difference was found in the second test for striking between snakes from Ozaukee and Racine when litter was nested within population. Snakes from Racine were the most likely to strike in the second antipredator test, and this time, exhibited the lowest amount of fleeing behavior. The same pattern in the fleeing behavior that occurred in the first antipredator test was present in the second test. Snakes from Milwaukee-Menard's Oak Creek fled the most, and the snakes from Milwaukee-Target Oak Creek fled less than snakes from all other populations except Racine.

Other Findings

Although my data indicated the existence of species differences, the low number of *T. radix* subjects could have led to this result. These data are inconsistent with the findings of Herzog et al. (1992), who found that South Dakota *T. radix* and Michigan *T. butleri* showed similar antipredator responses to one another. However, Arnold and Bennett (1984) found that head-tapping and tail-holding elicited defensive antipredator responses in *T. radix* from Illinois. It was interesting that none of the *T. radix* subjects exhibited the tail waving behavior, which could be a species-typical trait and would be interesting to examine in future research with more subjects.

The behaviors of the snakes in the first antipredator test were very similar to the behaviors in the second antipredator test. Only the number of tail waves significantly differed between the two tests.

Snakes were most likely to flee during the touching stimulus in both antipredator tests. This is consistent with what Albright (2001) found with *T. butleri*. The touching stimulus creates a higher threat level, which caused the subjects in this test to flee. In the second antipredator test, snakes were more likely to flee during the moving stimulus. Bowers et al. (1993) also concluded that the gartersnakes used in their experiment changed the type and intensity of their behavior as the levels of predatory threat increased.

Chemosensory Tests

Prediction 1

I did not find a difference in the between *T. radix* and Wisconsin *T. butleri* in the responses to the different stimuli, but there was a significant difference between the

responses of MI/OH *T. butleri* and Wisconsin *T. butleri* in their responses to fish. These data support my hypothesis that the Wisconsin *T. butleri* are more like *T. radix* than like MI/OH *T. butleri*.

Predictions 2, 3

In examining only the subjects from southeastern Wisconsin, snakes from the different counties did not differ in their responses to the worm and fish in the average of the two presentations. Although the differences were not significant, snakes from Ozaukee produced a higher TFAS to worm than did snakes from the other populations, whereas snakes from Waukesha-Vernon Marsh produced the highest TFAS to fish. However, when Wisconsin population differences were analyzed without the litter variable, significant population differences were found in the responses to worm and fish.

A strong indication of a difference among populations was found in the relative chemosensory results (W-F TFAS). Snakes from Ozaukee, Waukesha-Mitchell Park, and Milwaukee-Target Oak Creek preferred worms and snakes from Waukesha-Vernon Marsh, Milwaukee-Menard's Oak Creek, and Racine preferred fish. Snakes from the populations at the ends of the cline in southeastern Wisconsin (Ozaukee and Racine) preferred different prey items, indicating an overall difference in chemosensory preference. However, snakes from the populations in the middle of these areas did not exhibit a consistent variation in chemosensory preference.

Other Findings

The overall difference in the TFAS between the types of stimuli showed that the snakes were able to distinguish between water (the control) and the actual prey stimuli. The presence of order effects indicates that the responses to the stimuli were different,

depending on the order in which the stimuli were presented. The presence of the cotton swab itself, regardless of the prey (or control) type, could have elicited the tongue-flicking behavior upon the first presentation.

Attacks directed towards a stimulus have been described to indicate a stronger predatory response than tongue-flicks alone (Cooper & Burghardt 1990). In this experiment, more attacks were directed at the fish stimulus than the worm stimulus, something not seen by *T. butleri* in previous studies. *Thamnophis butleri* in Albright's study attacked the worm stimulus more often than the fish stimulus (Albright 2001). Lyman (1990) examined *T. butleri* from Michigan and found that eight of nine attacks were made to worm. Many of the snakes in this study, however, did not attack either stimulus and this might have been due to a different testing methodology in that snakes were not tested in their home cages. Differences were found in the number of attacks by snakes from different counties, with snakes from Racine county producing the greatest number of attacks, most of which were to the fish stimulus.

Although *Thamnophis* species often differ in their chemosensory preferences based on normal diet (Burghardt 1993), Burghardt (1969) found that responses made by *T. butleri* and *T. radix* were similar to each other and to the generalist *T. sirtalis*. The same conclusion was drawn by Burghardt after examining tongue-flicks to stimuli by *T. radix* and *T. butleri* (Burghardt 1967). Although the responses by *T. butleri* to fish, a prey item never eaten in the wild, may be the retention of a trait from its presumed generalist ancestor, it could be expected that some loss in the trait might have occurred in populations not undergoing gene exchange with *T. radix*.

Earlier research by Schwartz (1989) indicated differences between the

chemosensory responses of Michigan and Wisconsin *T. sirtalis*. Schwartz (1989) found that Wisconsin *T. sirtalis* responded more to fish extracts than did snakes from Michigan. Thus, ecological differences may also be affecting the chemosensory differences, which was a prediction of the local ecology hypothesis and not the main hypothesis of this study.

Prey Preference Test

Although not all snakes chose a prey item in the allotted time, the majority of snakes chose worm over fish. However, snakes from the counties in southeastern Wisconsin differed in their prey choices. Snakes from Ozaukee county chose worm first more often than did snakes from the other counties. Although Racine county snakes chose fish first more often compared to other counties, most of the Racine snakes still chose the worm over the fish.

Correlations among Behavioral and Morphological Measures

Negative correlations were found between mass, SVL, and body condition with water TFAS, prey attacks, and most strongly, worm TFAS and fish TFAS. Large responses to the water stimulus indicated an overall high responsivity to stimuli. Because this responsivity is correlated with mass, perhaps the snake's ability or need to search for prey items is dependent on its mass. If this is the case, smaller snakes may be more likely to search for prey, perhaps because of a greater need to eat earlier in life. Even so, mass, SVL, and body condition were most highly correlated with responses to the worm stimulus. This suggests that worms, the common species-typical diet, are most salient to hungry neonatal snakes. Waters and Burghardt (2005) examined the ontogeny of chemoreception in crayfish snakes and found that *Regina septemvittata*, which had

limited yolk and fat reserves, were more likely to show an increased interest in alternative prey types. *Regina grahamii* neonates, which had significantly more reserves, did not increase their responses to alternative prey items or even eat for many months after birth.

Another interesting correlation is the relationship between the number of strikes produced in antipredator behavior and the total number of chemosensory attacks given to a stimulus. Again, these behaviors could have something to do with responsivity.

However, I would have also expected a correlation between antipredator behavior and mass, which has been described previously in the snake literature (Langkilde et al. 2004).

Discriminant Function Analysis

The discriminant analysis yielded interesting results regarding the grouping of the populations in southeastern Wisconsin. When I conducted the analysis with all six populations, the percentage of groups classified correctly was significant, but fairly low (38%), indicating considerable variation within the populations. When I examined only Ozaukee and Racine populations, the percentage of correctly classified individuals almost doubled, which is consistent with the idea that snakes from these populations are very different. A high percentage of individuals were also correctly classified when I examined only Milwaukee and Waukesha populations, but this classification compared four populations, versus the two populations compared with the Ozaukee/Racine test. Still, the Milwaukee/Waukesha classification yielded stronger results above chance because the snakes were placed into four populations instead of two.

General Discussion

More significant differences among Wisconsin populations were found in the second antipredator test than in the first test. Snakes from Racine, the population furthest

south and therefore closest to the range of *T. radix*, showed greater levels of striking behavior than did snakes from Ozaukee, the population furthest from the range of *T. radix*. When only Racine and Ozaukee snakes were examined in the second antipredator test, Racine snakes were significantly more likely than Ozaukee snakes to strike. Ozaukee snakes were also more likely to flee than Racine snakes, although a significant difference was not found. Although significant differences were not found in all of the antipredator behaviors, snakes from Ozaukee and Racine showed a trend towards being the most different. Thus, I feel confident in concluding that the snakes from Racine were more aggressive and therefore behaved more like Illinois *T. radix* than did snakes from Ozaukee. Snakes from Ozaukee behaved more like *T. butleri* from other areas, as seen in past research with this species.

In feeding behavior, a difference in the number of attacks given to a stimulus was found between populations. Although many of the snakes did not attack either prey stimulus, the individuals that did attack a stimulus attacked the fish stimulus more often, something not previously observed. Snakes from Racine county elicited the greatest number of attacks, with most of them being towards fish. A significant difference was found between the Racine and Ozaukee snakes in the number of attacks to the fish stimulus.

In response to prey stimuli, the populations in southeastern Wisconsin did not differ significantly in the TFAS to worm and fish in the average of the two presentations. Although not significant, snakes from Ozaukee showed a higher TFAS to worm than did snakes from Racine. No discernable pattern was found in comparing the other populations in southeastern Wisconsin.

Perhaps the most important difference between Ozaukee and Racine populations was evident in the relative chemosensory preference analysis. In preference for a stimulus, using the average of the two presentations, snakes from Ozaukee preferred worms and snakes from Racine preferred fish. So, the snakes from the populations furthest north and furthest south differed in their chemosensory preferences. The snakes from the other populations in southeastern Wisconsin showed somewhat of a clinal pattern, except that snakes from Milwaukee-Target Oak Creek preferred worms. However, a large confidence interval indicated differences among the snakes in this population.

Choice of actual prey items indicated a preference for worms in all counties, although snakes from Ozaukee showed the greatest worm choice and snakes from Racine showed the lowest worm choice. However, snakes from Racine still chose worms over fish. Previous research has indicated that *T. radix* likewise prefer worms (Burghardt 1967, 1969).

Future behavioral research addressing the question of hybridization between these species should include more *T. radix* individuals and populations. Although the focus of this study was on *T. butleri* individuals, running the same tests with more *T. radix* individuals would lead to a greater confidence of the conclusions. It was difficult to compare past antipredator research with *T. radix* individuals because previous tests (Herzog et al. 1992; Arnold & Bennett 1984) were conducted in a different fashion. A greater number of *T. butleri* litters and populations from southeastern Wisconsin would also lead to greater confidence of the conclusions. A future study should especially include more litters from Ozaukee and Racine counties. Also, more litters from

Michigan should be added to the analyses. The Michigan *T. butleri* were only collected from one site. It is difficult to draw conclusions about a species based on one site within a larger geographic range.

Because *T. butleri* is considered threatened in southeastern Wisconsin, it is necessary to promote the conservation of this species. Since hybridization can ultimately lead to extinction (Levin 2002), the extent of the hybridization occurring in southeastern Wisconsin needs to be addressed further. The presence of hybridization has been documented in various animal species, most of which having occurred, at least in part, because of habitat degradation by humans or human-introduced invasive species (lizards: Capula 2002; deer: Abernethy 1994; fish: Dowling and Childs 1992; frogs: Schlefer et al. 1986).

Allendorf et al. (2001) listed several factors that need to be considered when assessing the value of a hybridized population, including how many pure populations of the taxon remain, the degree of differentiation between the hybrid and the pure populations, and whether or not the hybrid population poses a threat to the remaining pure populations.

My findings did corroborate with existing morphological and genetic data on the issue of gene flow between *T. butleri* and *T. radix* in southeastern Wisconsin. There does seem to be evidence for hybridization between these species based on behavioral data. Snakes from Ozaukee and Racine counties differed in many of the behaviors examined, but snakes from populations in Milwaukee and Waukesha counties did not exhibit any reasonable pattern. Preliminary genetic data indicate the presence of secondary contact of Wisconsin *T. butleri* with *T. radix* (Burghardt et al., unpubl. data). Further genetic

research is currently being conducted. Additional morphological measurements on the mothers of the individuals used in this study are also currently being obtained. These data will also aid in answering this long-standing taxonomic question and help with the conservation and management of both *Thamnophis butleri* and *T. radix* in southeastern Wisconsin.

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APPENDIX

Morphology

Tail length

When tail length was examined without SVL as a covariate, *T. radix* subjects were found to have longer tails than *T. butleri* snakes ($F_{1,171}=11.12$, $p=0.001$). When tail length was examined with SVL as a covariate, however, no significant species difference was found ($F_{1,171}=1.02$, $p=0.315$). When tail length was examined without SVL as a covariate, *T. radix* subjects had significantly longer tails than MI/OH *T. butleri* ($F_{1,46}=4.05$, $p=0.051$), but there was no significant difference in tail length between *T. radix* and MI/OH neonatal *T. butleri* when tail length was examined with SVL as a covariate ($F_{1,46}=0.04$, $p=0.847$). Similarly, *T. radix* subjects had significantly longer tails than Wisconsin *T. butleri* when tail length was examined without SVL as a covariate ($F_{1,137}=16.16$, $p=0.000$), but when tail length was examined with SVL as a covariate, there was no significant difference in tail length ($F_{1,137}=2.06$, $p=0.154$).

There were no significant differences in tail length between MI/OH and Wisconsin *T. butleri* when tail length was examined with or without SVL as a covariate (with: $F_{1,159}=0.48$, $p=0.490$; without: $F_{1,159}=0.54$, $p=0.464$). However, significant population effects were found among the populations of southeastern Wisconsin for tail length ($F_{5,7}=2.37$, $p=0.044$). The tail lengths of Racine snakes were significantly longer than snakes from Ozaukee ($p=0.000$).

Sex Differences

Sex differences were examined within each species. Significant sex effects were found in *T. radix* subjects for mass ($F_{1,8}=5.15$, $p=0.053$), but not for SVL ($F_{1,9}=0.29$, $p=0.603$) or tail length ($F_{1,8}=0.74$, $p=0.736$). SVL was used as a covariate for mass and

tail length. Females weighed significantly more than males, and although not significant, males were longer than females in both SVL and tail length.

In *T. butleri*, significant sex effects were found for SVL ($F_{1,142} = 5.12$, $p = 0.025$) and tail length ($F_{1,141} = 22.02$, $p = 0.000$), but not mass ($F_{1,141} = 0.84$, $p = 0.360$). SVL was used as a covariate for mass and tail length analyses. Males were longer in SVL and tail length than females, and although not significant, males had greater mass than females.

Litter Differences

Two *T. radix* litters were collected. Litter effects were found in *T. radix* subjects for SVL ($F_{1,9} = 36.87$, $p = 0.000$) and mass ($F_{1,8} = 7.99$, $p = 0.022$), but not for tail length ($F_{1,8} = 0.20$, $p = 0.666$). Thus, there were significant differences between the two litters in SVL and mass (see Table A.1 for summary morphology data by litter at birth).

Sixteen *T. butleri* litters were collected. I compared the litters within each population for mass, SVL, and tail length. Litter within population effects in *T. butleri* subjects were found for SVL ($F_{8,142} = 12.27$, $p = 0.000$), mass ($F_{8,141} = 26.40$, $p = 0.000$), and tail length ($F_{8,141} = 2.38$, $p = 0.020$). See Table A.2 for ANOVA results.

Growth

The paired t-tests revealed significant growth between the first and second measurements for mass ($t = -19.482$, $df = 164$, $p = 0.000$), SVL ($t = -16.911$, $df = 164$, $p = 0.000$), and tail length ($t = -7.257$, $n = 164$, $p = 0.000$).

No significant differences were found between the species in daily mass growth ($F_{1,164} = 0.39$, $p = 0.534$), SVL growth ($F_{1,164} = 0.48$, $p = 0.491$), or growth in tail length ($F_{1,164} = 0.00$, $p = 0.948$).

Table A.1. Summary morphology data by litter at birth for *Thamnophis butleri* and *T. radix*.

Population	Litter #	n		Mass (g)	SVL (mm)	Tail length (mm)	Condition index
Sterling (MI)	1	9	Mean	1.50	128.00	32.78	0.90
			Std. Dev	0.13	4.27	8.61	0.004
	2	9	Mean	1.15	125.11	36.89	0.83
			Std. Dev	0.31	14.85	5.23	0.003
Toledo (OH)	10	16	Mean	1.65	132.81	35.38	0.89
			Std. Dev	0.09	3.47	2.92	0.003
Mil-TOC	7	10	Mean	1.19	121.20	34.40	0.87
			Std. Dev	0.09	3.16	2.72	0.003
	8	8	Mean	1.42	122.25	35.25	0.92
			Std. Dev	0.13	5.29	3.11	0.003
Mil-MOC	6	13	Mean	1.32	126.92	34.54	0.86
			Std. Dev	0.17	6.92	4.08	0.003
Oza-CSL	22	8	Mean	1.52	133.88	34.75	0.86
			Std. Dev	0.23	5.67	2.71	0.003
	25	4	Mean	1.20	117.75	32.25	0.90
			Std. Dev	0.05	2.23	4.35	0.001
	27	11	Mean	0.86	114.64	30.73	0.82
			Std. Dev	0.05	1.91	2.00	0.001
Rac-CPP	14	14	Mean	1.55	132.07	37.07	0.88
			Std. Dev	0.16	5.70	2.62	0.002
	17	8	Mean	1.29	124.50	34.13	0.88
			Std. Dev	0.14	6.91	3.60	0.004
	18	10	Mean	1.25	127.00	38.20	0.85
			Std. Dev	0.15	5.21	1.93	0.001
	19	8	Mean	1.15	131.38	36.00	0.80
			Std. Dev	0.13	6.70	2.98	0.002
Wau-VM	20	15	Mean	0.82	115.00	30.87	0.81
			Std. Dev	0.08	3.91	4.75	0.002
	21	10	Mean	1.30	128.60	36.10	0.85
			Std. Dev	0.13	6.48	2.89	0.003
Wau-MP	5	8	Mean	1.34	128.50	35.63	0.86
			Std. Dev	0.12	6.35	3.02	0.002
Will (IL)	12	2	Mean	1.15	126.00	34.50	0.84
			Std. Dev	0.09	8.49	2.12	0.004
	13	10	Mean	1.92	157.00	39.70	0.79
			Std. Dev	0.12	6.00	3.23	0.002

Table A.2. ANOVA table of behaviors comparing litter within population effects in morphology measurements of *Thamnophis butleri*. P-values in bold face are statistically significant at $p \leq 0.05$ after using Holm's correction method for multiple comparisons.

Measurement	Litter (pop.)	F	df	P
Mass	Sterling (MI)	43.02	1	0.000
	Mil.-Target	21.41	1	0.000
	Ozaukee	23.33	2	0.000
	Racine	28.17	3	0.000
	Wauk.-Vernon	28.83	1	0.000
SVL	Sterling (MI)	0.91	1	0.341
	Mil.-Target	0.07	1	0.797
	Ozaukee	27.35	2	0.000
	Racine	4.58	3	0.004
	Wauk.-Vernon	28.73	1	0.000
Tail length	Sterling (MI)	9.43	1	0.003
	Mil. Target	0.03	1	0.872
	Ozaukee	0.71	2	0.492
	Racine	1.99	3	0.119
	Wauk.-Vernon	1.71	1	0.194

No significant sex differences were found in *T. radix* for daily mass growth ($F_{1, 11} = 0.07$, $p = 0.792$), SVL growth ($F_{1, 11} = 0.04$, $p = 0.855$) or tail length growth ($F_{1, 11} = 0.00$, $p = 0.950$). A significant sex difference was found in *T. butleri* for daily tail length growth ($F_{1, 151} = 5.84$, $p = 0.017$), but not for daily mass growth ($F_{1, 151} = 0.07$, $p = 0.795$) or SVL growth ($F_{1, 151} = 0.93$, $p = 0.336$). Males showed a significant increase in tail length.

A significant population difference was found in *T. butleri* subjects for daily mass growth ($F_{7, 152} = 9.82$, $p = 0.000$), daily SVL growth ($F_{7, 152} = 5.26$, $p = 0.000$), and daily tail length growth ($F_{7, 152} = 2.41$, $p = 0.023$). See Tables A.3, A.4, A.5 for ANOVA results.

Antipredator Behavior

Initial Antipredator Test- Sex Differences

Significant sex differences were found for strikes in *T. radix*, with females striking more often than males, but no significant sex differences were found for flees ($F_{1, 11} = 1.68$, $p = 0.224$) or flattenings ($F_{1, 11} = 1.46$, $df = 1$, $p = 0.255$). *Thamnophis butleri* subjects showed no significant sex differences for strikes ($F_{1, 158} = 1.54$, $p = 0.217$), flees ($F_{1, 158} = 0.01$, $p = 0.943$), flattenings ($F_{1, 158} = 0.26$, $p = 0.612$), or tail waves ($F_{1, 158} = 0.23$, $p = 0.634$). Although not significant, males performed more strikes, head or body flattenings, and flees than females, and females were more likely to tail wave.

Initial Antipredator Test- Litter Differences

Differences in behavior between the litters were analyzed with an ANOVA with litter nested within population (see Table A.6 for a summary data for antipredator behaviors by litter). Litter within population differences were found for strikes ($F_{9, 155} = 3.304$, $p = 0.001$), flees ($F_{9, 155} = 5.11$, $p = 0.000$), and tail waves ($F_{9, 155} = 2.72$, $p = 0.006$).

Table A.3. Statistical differences in daily mass growth in *Thamnophis butleri* subjects. All p-values ≤ 0.05 after Holm's correction are in bold face.

	Tol (OH)	Oz-CSL	Wa-MP	Wa-VM	Mi-MOC	Mi- TOC	Ra- CPP
Ster (MI)	0.000	0.000	0.000	0.003	0.000	0.114	0.000
Tol (OH)		0.996	0.998	0.043	0.983	0.008	0.945
Oz- CSL			1.000	0.214	1.000	0.047	1.000
Wa-MP				0.628	1.000	0.276	1.000
Wa- VM					0.540	0.987	0.186
Mi- MOC						0.188	1.000
Mi- TOC							0.035

Table A.4. Statistical differences in daily snout-vent length growth in *Thamnophis butleri* subjects. All p-values ≤ 0.05 after Holm's correction are in bold face.

	Tol (OH)	Oz-CSL	Wa-MP	Wa-VM	Mi-MOC	Mi- TOC	Ra- CPP
Ster (MI)	0.000	0.051	0.126	0.885	0.011	0.677	0.002
Tol (OH)		0.570	0.948	0.004	0.984	0.052	0.765
Oz- CSL			1.000	0.509	0.992	0.902	0.999
Wa-MP				0.601	1.000	0.882	1.000
Wa- VM					0.160	0.999	0.071
Mi- MOC						0.508	1.000
Mi- TOC							0.465

Table A.5. Statistical differences in daily tail length growth in *Thamnophis butleri* subjects. No p-values reached $p \leq 0.05$ after Holm's correction.

	Tol (OH)	Oz-CSL	Wa-MP	Wa-VM	Mi-MOC	Mi- TOC	Ra- CPP
Ster (MI)	0.022	0.046	0.405	0.413	0.041	0.446	0.281
Tol (OH)		1.000	0.999	0.767	1.000	0.891	0.281
Oz- CSL			1.000	0.927	1.000	0.977	0.919
Wa-MP				0.999	0.999	1.000	0.999
Wa- VM					0.836	1.000	1.000
Mi- MOC						0.925	0.823
Mi- TOC							1.000

Table A.6. Summary of antipredator data of first test (2 days post-partum) for *Thamnophis butleri* and *T. radix*.

Population	Litter #	n		Strikes	Flees	Flattenings	Tail waves
Sterling (MI)	1	9	Mean	0.00	23.00	0.00	0.67
			Std. Dev	0.00	11.52	0.00	1.12
	2	9	Mean	0.00	37.22	0.00	2.89
			Std. Dev	0.00	6.76	0.00	2.52
Toledo (OH)	10	16	Mean	1.31	30.56	0.13	1.38
			Std. Dev	3.14	7.34	0.34	1.82
Mil- MOC	6	13	Mean	0.00	22.69	0.00	0.23
			Std. Dev	0.00	7.38	0.00	0.83
Mil-TOC	7	10	Mean	0.00	13.90	0.00	1.00
			Std. Dev	0.00	9.45	0.00	1.25
	8	8	Mean	6.25	18.38	0.00	0.00
			Std. Dev	7.25	5.68	0.00	0.00
Oza-CSL	22	8	Mean	0.00	22.38	0.00	0.04
			Std. Dev	0.00	6.09	0.00	0.74
	25	4	Mean	2.00	15.25	0.25	0.75
			Std. Dev	4.00	7.41	0.50	1.50
	27	11	Mean	0.00	22.91	0.00	0.18
			Std. Dev	0.00	8.61	0.00	0.61
Rac-CPP	14	14	Mean	3.50	11.57	0.21	0.14
			Std. Dev	6.51	8.31	0.43	0.54
	17	8	Mean	5.38	25.75	0.38	1.00
			Std. Dev	10.06	9.07	0.74	1.20
	18	10	Mean	12.50	12.30	0.50	1.50
			Std. Dev	19.52	8.03	1.08	2.59
	19	8	Mean	0.00	21.00	0.00	0.50
			Std. Dev	0.00	6.44	0.00	0.54
Wau-VM	20	15	Mean	0.73	16.60	0.00	0.13
			Std. Dev	2.02	4.70	0.00	0.52
	21	10	Mean	0.00	20.90	0.00	1.20
			Std. Dev	0.00	7.53	0.00	2.70
Wau-MP	5	8	Mean	2.00	20.75	0.00	0.00
			Std. Dev	4.90	8.14	0.00	0.00
Will (IL)	12	2	Mean	20.00	11.00	0.00	0.00
			Std. Dev	28.28	0.00	0.00	0.00
	13	10	Mean	5.50	12.80	0.10	0.00
			Std. Dev	8.49	5.35	0.32	0.00

No significant difference was found for flattenings ($F_{9, 155} = 1.25$, $p = 0.266$). Table A.7 lists the results of the ANOVA tests.

Second Antipredator Test- Sex Differences

No significant sex differences were found in *T. radix* for strikes ($F_{1, 11} = 2.39$, $p = 0.153$), flees ($F_{1, 11} = 1.40$, $p = 0.265$), or flattenings ($F_{1, 11} = 1.50$, $p = 0.248$). None of the *T. radix* subjects performed any tail waves. Although the differences were not significantly different, females performed more strikes, flees, and flattenings than males. No significant sex differences were found in *T. butleri* subjects for strikes ($F_{1, 158} = 0.78$, $p = 0.379$), flees ($F_{1, 158} = 0.01$, $p = 0.943$), flattenings ($F_{1, 158} = 0.26$, $p = 0.634$), or tail waves ($F_{1, 158} = 0.23$, $p = 0.634$).

Second Antipredator Test- Litter Differences

Differences in behavior between the litters were analyzed with an ANOVA with litter nested within population (see Table A.8 for a summary data for antipredator behaviors by litter). In the second antipredator test, litter within population differences were found for strikes ($F_{9, 153} = 6.51$, $p = 0.000$), flees ($F_{9, 153} = 2.60$, $p = 0.008$), flattenings ($F_{9, 153} = 5.85$, $p = 0.000$), and tail waves ($F_{9, 153} = 2.41$, $p = 0.014$). Table A.9 lists the ANOVA results.

Chemosensory Tests

Sex Differences

A repeated measures analysis of variance did not show a significant sex effect in *T. radix* subjects for the responses to the different stimuli for the average of the two presentations ($F_{1, 10} = 1.03$, $p = 0.334$). No significant sex effects were found in *T. butleri*

Table A.7. ANOVA table of behaviors comparing litter within population effects in first antipredator test (2 days post-partum) for *Thamnophis butleri*. P-values in bold face a significant at $p \leq 0.05$ after Holm's correction for multiple comparisons.

Behavior	Litter (pop.)	F	df	P
Strikes	Sterling (MI)	0.00	1	1.000
	Mil.-Target	3.89	1	0.050
	Ozaukee	0.15	2	0.863
	Racine	5.88	3	0.001
	Wauk.-Vernon	0.07	1	0.788
	Will (IL)	7.84	1	0.006
Flees	Sterling (MI)	15.73	1	0.000
	Mil.-Target	1.54	1	0.217
	Ozaukee	1.59	2	0.207
	Racine	7.85	3	0.000
	Wauk.-Vernon	1.92	1	0.168
	Will (IL)	0.09	1	0.760
Tail waves	Sterling (MI)	11.77	1	0.001
	Mil. Target	2.35	1	0.001
	Ozaukee	0.25	2	0.777
	Racine	2.08	3	0.106
	Wauk.- Vernon	3.61	1	0.059
	Will (IL)	0.00	1	1.000

Table A.8. Summary of antipredator data of second test (22-25 days post-partum) for *Thamnophis butleri*.

Population	Litter #	N		Strikes	Flees	Flattenings	Tail waves
Sterling (MI)	1	9	Mean	0.00	26.44	0.00	0.00
			Std. Dev.	0.00	7.23	0.00	0.00
	2	9	Mean	0.00	29.67	0.00	1.11
			Std. Dev	0.00	4.33	0.00	1.97
Toledo (OH)	10	16	Mean	0.50	26.69	0.00	0.19
			Std. Dev	1.75	6.30	0.00	0.75
Mil-MOC	6	13	Mean	0.00	23.23	0.00	0.08
			Std. Dev	0.00	6.67	0.00	0.28
Mil-TOC	7	10	Mean	1.40	16.00	0.00	0.20
			Std. Dev	4.43	7.13	0.00	0.42
	8	8	Mean	1.25	17.38	0.00	0.00
			Std. Dev	1.58	5.57	0.00	0.00
Oza-CSL	25	4	Mean	0.00	15.75	0.00	0.75
			Std. Dev	0.00	8.06	0.00	1.50
	27	11	Mean	0.00	19.73	0.00	0.55
			Std. Dev	0.00	7.04	0.00	1.21
	22	8	Mean	0.00	23.43	0.00	0.00
			Std. Dev.	0.00	4.20	0.00	0.00
Rac-CPP	14	14	Mean	13.93	11.00	0.64	0.14
			Std. Dev	15.73	4.44	1.01	0.36
	17	8	Mean	11.50	18.75	0.13	0.00
			Std. Dev	11.90	5.45	0.35	0.00
	18	10	Mean	17.10	18.70	0.20	1.00
			Std. Dev	16.96	7.65	0.42	1.89
	19	8	Mean	0.14	18.43	0.00	0.57
			Std. Dev	0.38	7.61	0.00	1.51
Wau-VM	20	15	Mean	0.00	22.73	0.00	0.00
			Std. Dev	0.00	6.29	0.00	0.00
	21	10	Mean	0.00	17.00	0.00	1.10
			Std. Dev	0.00	3.27	0.00	1.29
Wauk-MP	5	8	Mean	2.50	20.63	0.00	1.25
			Std. Dev	7.07	7.65	0.00	1.75
Will (IL)	12	2	Mean	37.50	12.00	1.50	0.00
			Std. Dev	16.26	2.83	0.71	0.00
	13	10	Mean	3.80	15.30	0.10	0.00
			Std. Dev	8.66	6.04	0.00	0.00

Table A.9. ANOVA table of behaviors that showed significant litter within population effects in second antipredator test (22-25 post-partum) for *Thamnophis butleri*. P-values in bold-face are significant at $p \leq 0.05$ after Holm's correction for multiple comparisons.

Behavior	Litter (pop.)	F	df	P
Strikes	Sterling (MI)	0.00	1	1.000
	Mil.-Target	0.00	1	0.966
	Ozaukee	0.00	2	1.000
	Racine	7.94	3	0.000
	Wauk.-Vernon	0.00	1	1.000
	Will (IL)	34.77	1	0.000
Flees	Sterling (MI)	1.15	1	0.285
	Mil.-Target	0.21	1	0.649
	Ozaukee	1.91	2	0.152
	Racine	4.31	3	0.006
	Wauk.-Vernon	4.87	1	0.029
	Will (IL)	0.45	1	0.504
Flattening	Sterling (MI)	0.00	1	1.000
	Mil.-Target	0.00	1	1.000
	Ozaukee	0.00	2	1.000
	Racine	7.81	3	0.000
	Wauk.-Vernon	0.00	1	1.000
	Will (IL)	29.25	1	0.000
Tail waves	Sterling (MI)	5.78	1	0.017
	Mil.-Target	0.19	1	0.668
	Ozaukee	0.96	2	0.386
	Racine	2.08	3	0.105
	Wauk.-Vernon	7.55	1	0.007
	Will (IL)	0.00	1	1.000

subjects for the responses to the different stimuli for the average of the two presentations ($F_{1, 157} = 1.13$, $p = 0.290$).

Litter Differences

In the average of the two stimuli presentations, a significant litter effect was found for all three stimuli (water: $F_{9, 154} = 3.55$, $p = 0.000$; worm: $F_{9, 154} = 5.58$, $p = 0.000$; fish: $F_{9, 154} = 3.23$, $p = 0.001$). Table A.10 lists the chemosensory data for the average of the two stimuli presentations. Both significant and non-significant results of litter within population effects for the average of the two presentations are found in Table A.11.

Table A.10. Summary of chemosensory data of the average of the two chemosensory tests for *Thamnophis butleri* and *T. radix*.

Population	Litter #	n		Water TFAS	Worm TFAS	Fish TFAS
Sterling (MI)	1	9	Mean	0.74	1.98	1.88
			Std. Dev	0.43	0.46	0.67
	2	9	Mean	1.10	2.05	1.82
			Std. Dev	0.36	0.52	0.41
Toledo (OH)	10	16	Mean	0.75	1.60	1.66
			Std. Dev	0.36	0.67	0.47
Mil-TOC	7	10	Mean	0.82	1.51	1.31
			Std. Dev	0.52	0.43	0.58
	8	8	Mean	0.96	1.74	1.89
			Std. Dev	0.46	0.51	0.64
Mil-MOC	6	13	Mean	1.22	2.15	2.42
			Std. Dev	0.42	0.43	0.37
Oza-CSL	22	8	Mean	1.10	2.25	2.08
			Std. Dev	0.33	0.69	0.41
	25	4	Mean	0.88	2.11	1.97
			Std. Dev	0.39	0.66	0.17
	27	11	Mean	1.26	2.64	2.20
			Std. Dev	0.44	0.51	0.62
Rac-CPP	14	14	Mean	0.65	1.17	1.71
			Std. Dev	0.38	0.46	0.85
	17	8	Mean	1.53	1.57	2.30
			Std. Dev	1.20	0.82	0.92
	18	10	Mean	1.23	2.47	2.48
			Std. Dev	0.29	0.54	0.48
	19	8	Mean	0.82	1.98	1.89
			Std. Dev	0.30	0.61	0.58
Wau-VM	20	15	Mean	0.99	2.41	2.58
			Std. Dev	0.52	0.68	0.66
	21	10	Mean	1.35	2.04	2.23
			Std. Dev	0.31	0.35	0.44
Wau-MP	5	8	Mean	0.79	2.03	1.80
			Std. Dev	0.51	0.44	0.31
Will (IL)	12	2	Mean	1.59	3.24	3.14
			Std. Dev	0.48	0.37	0.59
	13	10	Mean	1.01	1.98	1.80
			Std. Dev	0.37	0.48	0.45

Table A.11. ANOVA table of chemosensory responses to stimuli that showed significant litter within population effects in the average of the two chemosensory presentations for *Thamnophis butleri* and *T. radix*. P-values in bold face are significantly different at $p \leq 0.05$ after Holm's correction for multiple comparisons.

Stimulus	Litter (pop.)	F	df	P
Water	Sterling (MI)	2.51	1	0.115
	Mil.- Target	0.41	1	0.525
	Ozaukee	1.02	2	0.364
	Racine	7.05	3	0.000
	Wauk.-Vernon	3.33	1	0.070
	Will (IL)	2.50	1	0.116
Worm	Sterling (MI)	0.09	1	0.764
	Mil.- Target	0.77	1	0.381
	Ozaukee	1.85	2	0.160
	Racine	11.43	3	0.000
	Wauk.-Vernon	2.69	1	0.103
	Will (IL)	8.71	1	0.004
Fish	Sterling (MI)	0.06	1	0.806
	Mil.- Target	4.63	1	0.033
	Ozaukee	0.26	2	0.774
	Racine	4.17	3	0.007
	Wauk.-Vernon	2.27	1	0.134
	Will (IL)	9.10	1	0.003

Vita

Lauren E. Kirby was born in Knoxville, TN on January 12, 1980. Lauren is the oldest of three, with a younger brother and sister. She and her family moved to Kingsport, TN before she started Kindergarten and have lived there ever since. Lauren graduated from Dobyens-Bennett High School in 1998. She then went on to the College of Charleston, in Charleston, SC where she received a B.S. in psychology in 2002. After finishing her Master's in psychology, she will attend the University of Alabama, where she will pursue a Master's degree in Elementary Education. There she will pursue a future as a science teacher, hoping to share her love of science and herps with younger generations.